AN ABSTRACT OF THE THESIS OF

Marina Levata-Jovanovic for the degree of Master of Science in Microbiology presented on May 2, 1995. Title: Characterization of Dairy Leuconostocs and Method to Use *Leuconostoc mesenteroides* ssp. *cremoris* to Improve Milk Fermentations.

Abstract approved: ___ William E. Sandine

Dairy leuconostocs are frequently used as aroma-producing bacteria in mesophilic cultures used as starters for milk fermentations. Sixty *Leuconostoc* strains from different culture collections were studied taxonomically for species identification. Based on morphological, physiological and biochemical tests, twenty of these strains were typed as *Leuconostoc mesenteroides* ssp. *cremoris* (*Leu. cremoris*). The objectives of this study were to acquire additional information on phenotypic features of *Leu. cremoris* strains, such as diacetyl reductase activity and citrate utilization in milk, and to select strains most useful for producing flavor compounds, primarily diacetyl, in dairy fermentations. Great strain variability within *Leu. cremoris* species was observed. Diacetyl reductase specific activity, indirectly measured by the rate of NADH oxidation, ranged from 0 to 1603 U/mg of cell protein. Most of the strains utilized citrate under neutral conditions, pH 6.5, without concomitant production of diacetyl or acetoin. Addition of citric acid after preincubation (24 hr, 28°C) resulted in significant production of diacetyl (2.8-75.3 µg/ml) and acetoin (162.1-764.4 µg/ml) by pure *Leu. cremoris* cultures during further incubation (18 hr, 28°C). Simple and direct gas liquid chromatographic analysis without prior processing was applied to quantify acetaldehyde, ethanol, diacetyl, acetoin and acetic acid in milk fermented with *Leu. cremoris* and *Lactococcus lactis* ssp. *cremoris* (*Lc. cremoris*).

Leu. cremoris 91404 was selected as an aroma producer in preparation of experimental buttermilk based on the following characteristics: low diacetyl reductase activity; citrate utilization and high diacetyl production under acidic conditions; growth characteristics and compatibility with *Lactococcus* strains. However, no diacetyl was detected in buttermilk made in the traditional commercial manner. Fortification of ripened buttermilk with sodium citrate resulted in significant increase in diacetyl and acetoin production during buttermilk storage (5°C for as long as two weeks). Surplus of citrate, low pH (pH 4.5-4.7), sufficient number of active non-growing aroma producers, air incorporation during curd breaking and low temperature storage facilitated citrate metabolism toward production and conservation of flavor during two weeks of storage. Incorporation of a ropy *Lc. cremoris* strain in starter culture significantly improved the texture and appearance of experimental buttermilk.

Survey of commercial buttermilks available in the Corvallis, OR market showed wide variation in concentrations of volatile compounds and in organoleptic characteristics. Manufacture of experimental buttermilk under different conditions revealed that simple modifications in the traditional manufacturing procedure, involving starter composition and delayed citrate fortification, would yield refreshing product with clean, aromatic, thick and carbonated properties.

Characterization of Dairy Leuconostocs and Method to Use *Leuconostoc mesenteroides* ssp. *cremoris* to Improve Milk Fermentations

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Characterization of Dairy Leuconostocs and Method to Use *Leuconostoc mesenteroides* ssp. *cremoris* to Improve Milk Fermentations

CHAPTER 1

Introduction

Leuconostoc species are desirable in certain fermented foods because of their involvement in flavor development and preservation. In fermented dairy products their major role is to metabolize citrate and produce carbon dioxide, adding to the texture of a variety of cheeses. What is more important, small amounts of diacetyl, an essential flavor compound in products such as cultured buttermilk, cottage cheese, sour cream and ripened cream butter, is formed in this process (Marshall,1987). Strains of *Leuconostoc mesenteroides* ssp. *cremoris* (*Leu. cremoris*), *Leuconostoc mesenteroides* ssp. *dextranicum* (*Leu. dextranicum*), and *Leuconostoc lactis* (*Leu. lactis*) are those most used in mixed and multiple strain starter cultures used in the dairy industry. To perform their function, dairy leuconostocs need to be combined with acid-producing *Lactococcus lactis* ssp. *lactis* (*Lc. lactis*) or *Lactococcus lactis* ssp. *cremoris* (*Lc. cremoris*) strains (Gilliland,1985). The associative growth of lactococci and leuconostocs, as well as the process of diacetyl formation by lactic acid bacteria, is poorly understood and therefore difficult to control during the manufacture of dairy products. The major defect of many cultured dairy products is lack of flavor (Vedamuthu,1994). Scarce information on metabolism of *Leuconostoc* species and large variation between strains, even within one species (Hugenholtz,1993; Cogan and Jordan,1994), directed our attention to *Leuconostoc* strains maintained in the Oregon State University (OSU) dairy culture collection. The objectives of this study were: (i) to identify and characterize *Leu. cremoris* strains in the OSU culture collection of dairy leuconostocs, and (ii) to produce refreshing, flavorful, carbon dioxide-containing buttermilk using selected strains of lactococci and *Leu. cremoris.*

Chapter 2 is the review of the literature regarding leuconostocs and application of dairy leuconostocs in the production of a variety of cultured dairy products.

Chapter 3 details the survey of sixty *Leuconostoc* strains originating from different collections and commercial dairy products. Based on morphological, physiological and biochemical tests, twenty strains were typed as *Leu. cremoris.* In addition to taxonomic criteria traditionally used to define *Leu. cremoris* (Garvie,1984), strains were examined for citrate utilization, diacetyl and acetoin production under neutral and acidic conditions, level of diacetyl reductase activity, plasmid profiles, total soluble cell protein patterns and growth compatibility with lactococci.

In Chapter 4, gas-liquid chromatographic (GLC) analyses of volatile compounds produced by *Leu. cremoris* is described. Direct injection of culture on a GLC column and successive GLC detection of acetaldehyde, ethanol, diacetyl, acetoin, and acetate produced in milk was found to be a simple and exact method to analyze volatile end products of *Leuconostoc* metabolism. Based on growth and biochemical characteristics, strain 91404 was selected for further mixed culture studies directed to improve milk fermentations. GLC analyses showed that addition of citrate to cultured buttermilk after ripening, i.e. when the coagulum was stirred, enhanced the concentrations of diacetyl and acetoin produced by *Leu. cremoris.*

Chapter 5 describes analyses of buttermilk samples obtained from six regional dairies over a one year period. GLC analyses of volatile compounds revealed a wide variation in commercially available buttermilk, and often a lack of well-balanced culture flavor. Flavorful buttermilk made in our laboratory with selected *Leu. cremoris* and *Lc. cremoris* strains, involved the addition of citrate after fermentation. The process was evaluated commercially with successful results.

CHAPTER 2

Literature Review

2.1 Taxonomy of the genus *Leuconostoc*

Bacteria of the genus *Leuconostoc* have been found in a great number of natural and man-made habitats. Numerous strains have been isolated from plant matter, fermenting vegetables, silage, milk, dairy products, wines (Garvie,1960; Whittenbury,1966; Garvie and Farrow,1980; Garvie,1984; Izuagbe et al.,1985; Gilliland,1985; Fantuzzi et al.,1992), and from chilled-stored and vacuum-packed meats (Shaw and Harding,1984, Shaw and Harding,1989, Grant and Patterson,1991). *Leuconostoc* species, which were previously thought to be of no importance in human or animal health were recently isolated from human blood, and related to serious infections (Isenberg et al.,1988; Barreau and Wagener,1990; Golledge,1991). Leuconostocs are normally found in the same habitats as lactococci and lactobacilli. Non-acidophilic leuconostocs are not easily separated from heterofermentative lactobacilli, e. g. *Lactobacillus confusus* and *Lactobacillus viridescens*, because of their similar biochemical and physical characteristics. Actually, comparative analysis of 16S rRNA sequences showed that species of these two genera form a natural grouping, which can be termed the "leuconostoc branch" of the lactobacilli (Yang and Woese,1989). Only the acidogenic species, *Leuconostoc oenos*, and its rRNA show features typical of a rapidly evolving organism with unusual or atypical phenotypic properties, that set it apart from other members of this natural group. *Leuconostoc oenos* is the most easily recognized species because it is acidophilic and ethanol tolerant (Van Vuuren and Dicks,1993).

The taxonomy of the *Leuconostoc* has recently undergone drastic modification (Table 2.1), and more changes may be expected in the future, indicating how neglected this group has been since the work of Garvie in the early 1980's.

Table 2.1 Current *Leuconostoc* species.

Species	Literature cited
1. *Leu. mesenteroides* ssp. *mesenteroides*	Garvie, 1983
Leu. mesenteroides ssp. *dextranicum*	Garvie, 1984
Leu. mesenteroides ssp. *cremoris*	Garvie, 1986
2. *Leu. lactis*	Garvie, 1984, 1986
3. *Leu. oenos*	Garvie, 1967a, 1986
4. *Leu. paramesenteroides*	Garvie, 1984, 1986
5. *Leu. pseudomesenteroides*	Farrow et al., 1989
6. *Leu. citreum*	Farrow et al., 1989
7. *Leu. gelidum*	Shaw& Harding, 1989
8. *Leu. carnosum*	Shaw& Harding, 1989
9. *Leu. fallax*	Martines-Murcia, 1991
10. *Leu. argentinum*	Dicks et al., 1993

In 1983 Garvie proposed that *Leu. mesenteroides* include, as subspecies, those strains previously classified as *Leu. mesenteroides*, *Leu. dextranicum*, and *Leu. cremoris* (Garvie,1983), thus reducing the number of species in the genus from six to four. In the last few years six new species were added to the genus *Leuconostoc*. Table 2.1 lists the four classical and six recently recognized species. In Bergey's *Manual of Systematic Bacteriology* (1986), leuconostocs have been reassigned from the family *Streptococcaceae* to the family *Dienococcaceae.*

Taxonomic studies have been based mostly on carbohydrate metabolism, physiological tests, cellular fatty acid composition, numerical analyses of total soluble cell proteins, lactate dehydrogenase profiles, and DNA homology with type strains

(Garvie,1976; Dicks et al.,1990; Shaw and Harding,1989; Picks and Van Vuuren,1990; Dicks et al.,1993). In 1989, Schillinger et al. identified a new species, *Leu. amelibiosum* using DNA-DNA and DNA-rRNA hybridization techniques. However, in 1992 this strain was found to be identical with *Leu. citreum*, and the new species designation was abandoned (Takahashi et al.,1992). Also, it was recently proposed that *Leuconostoc paramesenteroides* and related species be reclassified in a new genus *Weissella* (Collins et al.,1993).

2.2 Dairy leuconostocs

Considering definition that only species involved in dairy fermentation should be specified as dairy leuconostocs, only species *Leu. mesenteroides* and *Leu. lactis* should be included (Dessart and Steenson,1995). Cultures of *Leu. mesenteroides,* primarily subspecies *cremoris*, and *Leu. lactis*, are frequently used together with *Lactococcus* species as mesophilic starter cultures in dairy fermentations (Gilliland,1985). Except for *Leu. lactis*, most leuconostocs grow poorly in milk. For lactose-positive strains this is probably the result of their inability to produce sufficient proteinase to hydrolyze milk proteins to the amino acids and small peptides required for growth (Garvie,1960). An extracellular proteolytic system has never been identified for leuconostocs, while intracellular peptidases consisting of dipeptidases and aminopeptidases have been characterized by El-Shafei et al. (1990). *Leuconostoc* bacteria are very fastidious in their growth requirements. A number of amino acids, and growth factors such as purines, pyrimidines, riboflavin, pyridoxal, and folic acid are required for their growth (Garvie,1967b). Supplementation of media with yeast extract, as a rich source of amino acids and peptides, stimulates the growth of leuconostocs in milk (Cogan,1975; Gilliland,1985). The most commonly used laboratory media for the propagation of leuconostocs are MRS broth (de Man et al.,1960) and Elliker's lactic broth (Elliker et al.,1956), both of which support luxuriant growth of leuconostocs. Most selective media for enumeration of leuconostocs in mixed cultures are based on citrate utilization (Billie et al.,1985). Benkerroum et al. (1993) formulated a selective medium

(LUSM) for the isolation of *Leuconostoc* spp. from vegetables and dairy products using vancomycin, tetracycline, sorbic acid, sodium azide, and cysteine hydrochloride as selective agents. Differential counting procedures using vancomycin as the selective agent have proven satisfactory since vancomycin resistance in leuconostocs is well documented (Orberg and Sandine,1984; Simpson et al.,1988; Faclam et al.,1989; Farrow et al.,1989). Some other characteristics of the genus *Leuconostoc* are listed in Table 2.2.

Dairy leuconostocs, mainly *Leu. cremoris* and *Leu. lactis* , are primarily important for flavor generation in cultured dairy products (Vedamuthu,1994). The production of flavor by *Leuconostoc* is mainly due to synthesis of diacetyl during metabolism of citrate.

Citrate metabolism in the dairy *Leuconostoc*

In the past, *Lactococcus lactis* ssp. *lactis* biovar. diacetylactis (*Lc. diacetylactis*) has been used to study citrate metabolism (Seitz et al.,1963b; Harvey and Collins,1963; Speckman and Collins,1968), and the findings are presumed to be transferable to leuconostocs. Cogan and Jordan (1994) have shown that this approach is fundamentally wrong because sugar and organic acid metabolism by leuconostocs is quite different from that of lactococci.

Leuconostocs, being heterofermentative lactic acid bacteria, produce flavor compounds such as lactate, acetate, ethanol and carbon dioxide, as the end products of sugar fermentation. The production of diacetyl is possible only if an additional source of pyruvate is present. Citrate metabolism provides a means of producing "surplus" pyruvate.

Table 2.2 Characteristics of genus *Leuconostoc.*
(Garvie,1984; Garvie,1986; Milliere et al.,1989)

Gram positive coccoid cells
Occur in pairs and chains
Non motile
Non-spore forming
Catalase negative
Cytochrome oxidase negative
L-Arginine dihydrolase negative
Facultative anaerobes
Nitrates not reduced
Indole not formed
Non-hemolytic
Non-proteolytic
No tricarboxylic acid cycle
Heterofermentative (fermentation of glucose via phoshoketolase pathway)
D-(-) lactate exclusively produced (>95%)
Vancomycin resistant
Chemoorganotrophs (require nicotinic acid, thiamin, biotin, and pantothenic acid or one of it's derivatives)
Optimal temperature 20-30°C
No growth at 45°C
pH optimum near neutrality (except for *Leu. oenos)*
Litmus milk weakly acidified or not changed (except *Leu. lactis*)
Milk is not clotted without added yeast extract
Some species produce dextrane from sucrose
Some species metabolize citrate in presence of fermentable carbohydrate
Generally regarded as non-pathogenic

Presence of citrate permease is essential for metabolism of citrate. Citrate transport in leuconostocs is encoded on a plasmid (Lin et al.,1991), which explains the instability of citrate utilization (Fantuzzi et al.,1991). The permease of *Leuconostoc* was found to be homologous to the *Lc. lactis* permease (David et al.,1990). In all citrate-utilizing lactic acid bacteria citrate is converted initially to oxalacetate and acetate by the enzyme citrate lyase. Mellerick and Cogan (1981) showed that citrate lyase is inducible in *Leuconostoc.* Oxalacetate is further decarboxylated to pyruvate. Besides the formation of acetate and CO_2 in the initial breakdown of citrate, and production of lactate as primarily a product of pyruvate reduction, the compounds acetoin, diacetyl and butanediol are often produced. However, the exact sequence of reactions leading to the production of these compounds is still a matter of debate. Two mechanisms have been proposed (Fig 2.1): (i) condensation of acetyl-coenzyme A with acetaldehyde-thiamin pyrophosphate catalyzed by the enzyme diacetyl synthetase (Speckman and Collins,1968), and (ii) oxidative decarboxylation of α-acetolactate, an intermediate of citrate metabolism (Seitz et al.,1963b; Starrenburg and Hugenholtz,1991; Verhue and Tjan,1991). α-Acetolactate is synthesized from two pyruvate molecules catalyzed by the inducible enzyme acetolactate synthase, and subsequently enzymatically decarboxylated to acetoin (Hugenholtz and Starrenburg,1992). Acetoin is either excreted as an end product or is reduced to butanediol by the enzyme acetoin reductase. In this sequence of reactions, diacetyl is only produced as a byproduct resulting from chemical (aerobic) decarboxylation of α-acetolactate (Jordan and Cogan,1988). Once diacetyl is formed by the culture, it is susceptible to degradation to acetoin by diacetyl reductase (Seitz et al.,1963a; Hugenholtz,1993). Recent studies with *Lc. lactis* have demonstrated that the same enzyme is responsible for both irreversible reduction of diacetyl, and reversible reduction of acetoin (Crow,1990). Acetoin production may provide the way of removing excess toxic pyruvate from the cell (Collins,1972), while the reductive reactions play a physiological role in regenerating the cofactor (NAD(P)).

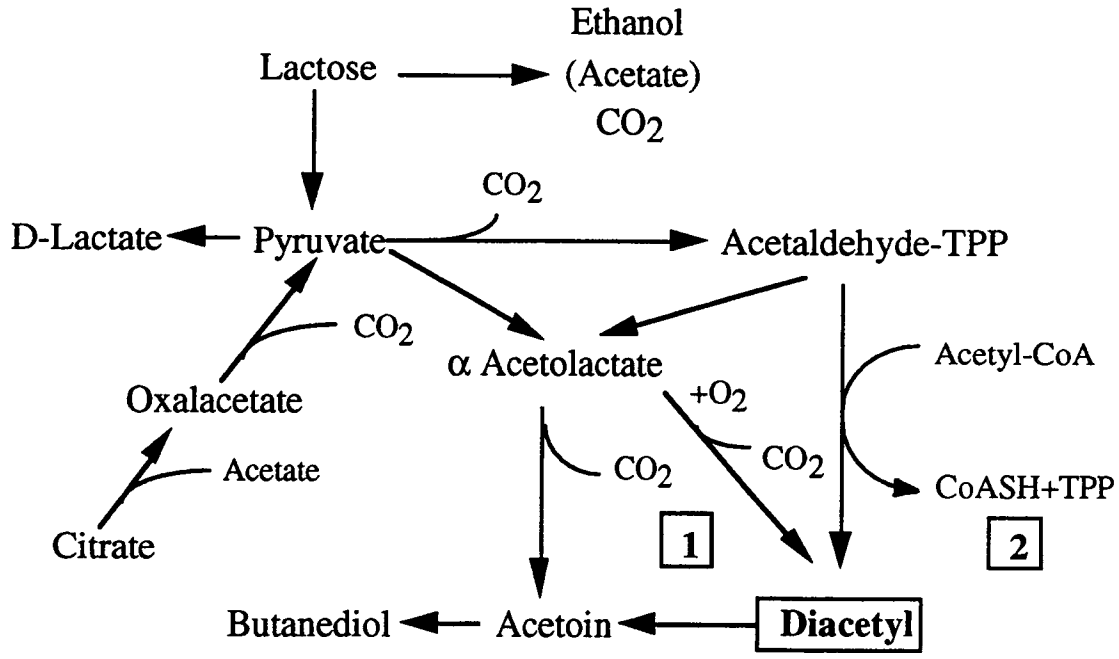

Figure 2.1 Schematic presentation of lactose and citrate metabolism in *Leuconostoc* ssp. according to (1) Starrenburg and Hugenholtz (1991) and (2) Collins (1972).

Furthermore, the end products of the reduction reactions are flavorless and odorless (Gilliland,1985), and the knowledge of the biochemical pathways leading to flavor production can help in selecting appropriate starter cultures. Most research on citrate metabolism and diacetyl production has been limited to studying the influence of several environmental and biological factors such as: pH (Cogan et al.,1981); oxygen tension (Kaneko et al.,1990; Bassit et al.,1993); the presence or absence of lactose (Drinan et al.,1976), glucose (Cogan,1987), metal ions (Kaneko et al.,1987); growth phase and the type of lactic acid bacteria (Marshal,1987; Hugenholtz and Starrenburg,1992). Metabolic studies using continuous cultivation under controlled conditions have been reported for *Leuconostoc* spp. (Schmitt et al.,1990), providing valuable data on the energetics of citrate utilization and the relationship between citrate metabolism and acetoin and/or diacetyl production. Genetic information on citrate metabolism in lactic acid bacteria (Kempler and McKay,1981; Sesma et al.,1990; Silke et al.,1990) and studies of molecular genetics of

dairy leuconostocs (Wyckoff,1992) can be used to control and improve diacetyl production for dairy fermentations.

Use of dairy leuconostocs in cultured milk products

Dairy leuconostocs are used as flavor producers in the production of cultured buttermilk, cream butter, sour cream, creamed cottage cheese and cream cheese. Dairy leuconostocs are also responsible for eye formation through CO_2 production from citrate and lactose in many cheese varieties, such as Edam and Gouda.

Among the various cultured dairy products produced in the United States, cultured buttermilk shows the widest possible variation in quality (Keenan et al.,1968; Vedamuthu, 1985). Cultured buttermilk is a mildly acid, smooth liquid milk drink with a delicate flavor contributed primarily by diacetyl, lactic acid, carbon dioxide, ethanol and acetic acid. The most common defect in buttermilk is lack of buttery flavor, i.e. lack of sufficient diacetyl (Vedamuthu,1985) which can be attributed to improper starter cultures, poor quality raw material or to the fermentation process itself (Lundstedt and Corbin,1983). Another serious problem is development of "green apple" or "yogurt like" off-flavor that is attributed to a relatively high acetaldehyde concentration in proportion to the diacetyl (Lindsay et al.,1965).

Commercial starter cultures for buttermilk may contain *Lc. lactis* or *Lc. cremoris* as acid producers, and *Leu. cremoris* or/and *Lc. diacetylactis*, as citrate utilizers and flavor producers (Tamime and Robinson,1988). Associative growth of lactococci and leuconostocs in mixed cultures is symbiotic, and their functional relationship is synergistic (Vedamuthu,1994). Lactococci produce stimulatory substances necessary for growth of leuconostocs (Boquien et al.,1988) and provide the acidic environment required for biosynthesis of diacetyl (Cogan et al.,1981). The stimulation of growth may be due to amino acid production by lactococci since *Leuconostoc* strains are unable to hydrolyze milk proteins and liberate amino acids required for growth. The advantage of using leuconostocs in mixed starters is their ability to reduce acetaldehyde to ethanol and eliminate the "green"

off-flavor (Keenan et al.,1966). Because of the complex interrelationship between growth rate and citrate utilization in mixed cultures, it is necessary to select compatible strains of the two groups to promote their balanced growth. Leuconostocs must be permitted to reach sufficiently high numbers to be able to carry out the citrate metabolism before the pH drops to a low level (Walker and Gilliland,1987). Incubation between 21 and 25°C permits leuconostocs to reach a high population, while at the same time growth of the metabolically more active lactococci is slowed (Pack et al.,1968b; Goel and Marth, 1969; Cooper and Collins,1978). Beside selection of compatible strains, various modifications of procedures for manufacture of buttermilk have been investigated. Fortification of milk with citrate (Mather and Babel,1959), cooling of the culture at the peak of diacetyl concentration (Pack et al.,1968b), incorporation of air by agitation (Prill and Hammer,1939; Bassit et al.,1993), and hydrogen peroxide-catalase milk treatment (Pack et al.,1968a) are examples of procedures applied to enhance and stabilize the diacetyl content in buttermilk.

CHAPTER 3

Identification of *Leuconostoc mesenteroides* ssp. *cremoris* strains

3.1 Introduction

Species of the genus *Leuconostoc* are commonly isolated from a variety of habitats such as plant material and dairy products (Garvie,1960; Whittenbury,1966). Strains of taxa *Leuconostoc mesenteroides* ssp. *mesenteroides (Leu. mesenteroides)*, ssp. *dextranicum* (*Leu. dextranicum*), ssp. *cremoris* (*Leu. cremoris*), and *Leuconostoc lactis* (Garvie,1983; Garvie,1986) are frequently used, together with *Lactococcus* ssp., as mesophilic starter cultures in dairy fermentations. The ability of *Leu. cremoris* to produce diacetyl from citrate has led to its widespread use as a characteristic aroma producer in cultured dairy products, such as cultured buttermilk, creamery butter, cultured sour cream, and certain cheeses (Vedamuthu,1994)). Their heterofermentative metabolism is useful for certain open textured cheese specialities (Gilliland,1985), while their ability to reduce acetaldehyde to ethanol (Lindsay et al.,1965; Keenan et al.,1966) make *Leuconostoc* species favorable for eliminating the "green" flavor defect in buttermilk. However, use of inappropriate starters often causes lack of a well-balanced flavor in many cultured dairy products.

The objectives of this study were to: (i) identify *Leu. cremoris* strains in our culture collection based on morphological, physiological and biochemical tests (Garvie,1984; Milliere et al.,1989); (ii) acquire additional information on the phenotypic properties of *Leu. cremoris* strains obtained from different universities and commercial collections and (iii) select the strains most useful in dairy fermentations to produce flavor compounds.

3.2 Material and Methods

Organisms and growth conditions

Leuconostoc and *Lactococcus* strains used in this study were from the stock culture collection in the Dairy Microbiology Laboratory at Oregon State University, Corvallis, OR. The twenty strains, identified as *Leu. cremoris* , were originally obtained from different universities and commercial laboratories. Their sources are listed in Table 3.1. *Leu. cremoris* strain 19254 was included as a reference strain and was obtained from the American Type Culture Collection (ATCC) in Rockville, MD.

Table 3.1 *Leuconostoc mesenteroides* ssp. *cremoris* strains used in this study.

Strain	Source	Strain	Source
1039	Valio Finish Coop.	L-5-319	Iowa State University
1040	Valio Finish Coop.	122-5	Iowa State University
1041	Valio Finish Coop.	173	Oklahoma State University
Lfa6	Valio Finish Coop.	176	Oklahoma State University
Sepa1	Valio Finish Coop.	J	Oklahoma State University
P1	Valio Finish Coop.	A18	Oklahoma State University
CAF7	Purdue University, IN	CAE	Oklahoma State University
91404	Moseley Lab, IN	Da3	Danish Starter, Oregon State University
44-4	E. R. Vedamuthu	Da15	Danish Starter, Oregon State University
30	E. Lundstedt	104	Oregon State University

Leuconostoc cultures were grown in MRS broth (55g/l of dehydrated MRS broth; Difco, Detroit, MI) supplemented with 0.1% sodium citrate for 24hr at 28°C, unless otherwise stated. Cultures were maintained in MRS broth at 4°C and frequently subcultured. Three percent of *Leuconostoc* cultures was used as inoculum in all experiments.

Lactococcus strains used in mixed-strain studies were *Lactococcus lactis* ssp. *lactis* (*Lc. lactis*) C2, ML3, 197, and LM2301, *Lactococcus lactis* ssp. *cremoris* (*Lc. cremoris*) 205, 224, HP, 352, 188, and 203. *Lc. lactis* ssp. *lactis* biovar. diacetylactis (*Lc. diacetylactis*) 18-16 and its mutant EmI were used for comparison. *Lactococcus* strains used as indicator organisms in well diffusion assays were: *Lc. lactis* 1363, ML3, C2, and *Lc. cremoris* 205 and 224. *Lc. lactis* ATCC 7962 was used as nisin producer in these experiments. Lactococci were propagated in M17G (M17 with 0.5% glucose) (Terzaghi and Sandine,1975)) and in 11% reconstituted skim milk (RSM) using 1% inocula and incubation at 28°C for 24 hr. Lactococci were maintained at 4°C in 11% RSM without previous incubation and later incubated (18 hr at 22°C) as needed.

Stock cultures of leuconostocs and lactococci were stored at -70°C in sterile 11% RSM supplemented with 20% (v/v) glycerol.

Micrococcus luteus (*M. luteus*) was used as the indicator strain in a standard bioassay procedure for detecting possible antimicrobial activity of *Leu. cremoris* strains. *M. luteus* was maintained on slopes of YGM assay agar containing (g/l): bacteriological peptone, 10; beef extract, 3; sodium chloride, 3; yeast extract, 1.5; glucose, 5; and agar, 15; pH 7.4. *M. luteus* was subcultured every two weeks and stored at 4°C after incubation at 28°C for 48 hr.

Morphological, physiological and biochemical tests

Cell morphology was assessed using the Gram stain. Carbon dioxide production from glucose was detected using inverted Durham tubes in MRS broth, pH 6.5 (Difco, Detroit, MI), modified by the omission of citrate. Catalase activity was detected from smears on glass slides of leuconostocs grown aerobically on MRS agar, after addition of two drops of 20% H_2O_2 (v/v).

Growth at 37, and 45°C, in MRS broth was monitored by measuring the absorbance at 600 nm using a model DU40 spectrophotometer (Beckman, Seattle, WA). Growth of *Leu. cremoris* cultures in 11% RSM, at 22 and 28°C was screened using the method of Kanasaki et al. (1975), modified as follows: 0.5 ml of sample, periodically removed from the milk culture, was added to 4.5 ml of 0.2% EDTA, pH 12.5. After slight vortexing, the absorbance was read immediately at 410 nm using a Perkin-Elmer, model 35 Spectrophotometer. A mixture of EDTA, pH 12.5, and uninoculated 11% RSM was used as the blank. Growth patterns at each temperature was examined by plotting the absorbance vs. time.

Reactions in litmus milk were examined for acid production, clot formation and reduction after 24 hr incubation at 28°C. Acidity (pH), diacetyl and acetoin production by pure cultures were followed in 11% RSM, then in milk supplemented with 0.5% glucose, in milk with 0.2% citrate, and in milk with 0.3% yeast extract. Nonfat-dry-milk (NFDM), which was used for preparation of RSM, contained 0.1% citrate, as analyzed by method of Marier and Boulet (1958). The pH of the cultures was measured with a Corning Model 125 pH meter.

The production of acids from 49 carbohydrates was tested using the API 50 CH Test System (API S.A., Montalieu-Vercieu, France) according to the manufacturer's instructions. Dextran production was tested on 5% (w/v) sucrose agar (Garvie,1984). Niven's arginine broth (Niven et al.,1941) was used to detect L-Arginine dihydrolase

activity. The type of lactic acid formed in diluted tomato broth (DTB) (Garvie,1984) was determined enzymatically by kit obtained from Boehringer/Mannheim, GmbH.

Vancomycin resistance was tested on vancomycin-containing MRS agar. Stock solutions (10 mg/ml) of vancomycin hydrochloride (Sigma Chemical Co., St.Louis, MO), were filter sterilized using 0.45 µm pore diameter filters (Nalge Co., Rochester, NY), and stored at -20°C. Vancomycin solution was added to MRS agar immediately before use to a final concentrations of 30 µg/ml or 50µg/ml. MRS agar containing 30 µg/ml of vancomycin was also used to selectively inhibit the growth of lactococci and therefore allowed direct enumeration of *Leu. cremoris* in multiple-strain cultures. Growth of *Leu. cremoris* strains in presence of selective agents: sodium azide, vancomycin, and tetracycline (Sigma Chemical Co., St.Louis, MO) was tested on LUSM medium that is suitable for isolation of leuconostocs (Benkerroum,1993).

Citrate utilization was performed on the citrate indicator agar (Kempler and McKay,1980) supplemented with 0.3% yeast extract, and on PMN agar (Walker and Gilliland,1987) supplemented with indicators: potassium ferricyanide,0.1%; ferric citrate, 0.025%; and sodium citrate, 0.025%.

Phenotypic characterization of *Leuconostoc mesenteroides* ssp. *cremoris*

Strains that were identified as *Leu. cremoris* were further characterized for: (a) citrate utilization, and diacetyl and acetoin production under neutral and acidic conditions; (b) diacetyl reductase activity; (c) plasmid profiles; (d) total soluble cell protein patterns; (e) production of antimicrobial substances, and (f) compatibility with lactococci as follows:

(a) Citrate utilization by *Leu. cremoris* in 11% RSM supplemented with sodium citrate to 0.2%, was determined using an enzymatic analysis kit (Boehringer/ Mannheim, GmbH). Diacetyl and acetoin production by pure *Leu. cremoris* strains under acidic conditions was tested using a modified method of Mather and Babel (1959). This procedure consisted of growing *Leu. cremoris* strains in 11% RSM for 24 hr at 28°C, and

acidifying the milk cultures with 1M citric acid to pH 4.3, followed by incubation for an additional 18 hr. Simultaneously, samples were acidified with 2M lactic acid to pH 4.3 used as a control. Diacetyl and acetoin produced in these milk cultures were qualitatively detected (King,1948).

(b) Crude cell-free extracts of *Leu. cremoris* were tested for the presence of diacetyl reductase (DR) and NADH oxidase activity, and were prepared as follows: Overnight cultures grown in 200 ml of MRS broth were harvested by centrifugation at 5,000 rpm for 10 min at 4°C, washed twice in 0.85% (w/v) NaCl and resuspended in approximately 1 ml of 0.2M phosphate buffer, pH 7.0. These cells were disrupted in 2ml microfuge vials (National Scientific Supply Company, Inc., San Rafael, CA) with zirconium beads, 0.1 mm in diameter, using a Mini-Bead Beater (Biospec Products, Bartlesville, OK). Cell suspensions were held on ice and agitated violently five times for 30 sec. Cell debris was removed by centrifugation at 13,000 rpm in an IEC Spinette centrifuge for 10 min at 4°C. The resulting cell-free extracts were stored frozen at -20°C until used. Protein concentrations were determined by the method of Lowry et al. (1951). The activity of DR (EC 1.1.1.5) was derived by subtracting the activity of NADH oxidase from the total, apparent activity of DR. The reaction mixture for the assay of total DR activity contained 0.5 µmol NADH, 20 µmol diacetyl, 0.1M phosphate buffer, pH 6.0, and 0.2 ml cell extract in a total volume of 1 ml. The reaction mixture for the assay of NADH oxidase was the same as that for DR except that diacetyl was omitted. The reaction was started by the addition of cell extract, and the rate of NADH oxidation was measured by recording the initial decrease in absorbancy at 340 nm with a Beckman Model DU-40 spectrophotometer at room temperature. One unit of enzyme activity was defined as the amount of enzyme oxidizing one µmol of NADH per minute. A molar extinction coefficient of 6.22×10^3 $M^{-1}cm^{-1}$ at 340 nm for NADH was used to calculate the activity. Specific activities of DR and NADH oxidase were calculated in units per milligram of protein.

(c) Plasmid DNA was isolated from *Leu. cremoris* strains using the procedure described by Anderson and McKay (1983) with the modification suggested by Wyckoff et al. (1991). Proteinase K used in this procedure was provided by Sigma Chemical Co. (St. Louis, MO). *Escherichia coli* V517 plasmids (Macrina,1978) were used as molecular size standards.

(d) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of soluble proteins in cell extracts of *Leu. cremoris* strains was performed using the method described by Hames and Rickwood (1990). Gels were stained with Coomassie blue. Low range protein ladder (BioRad, Richmond, CA) was used as molecular weight standard.

(e) To screen eventual production of antimicrobial substances by *Leu. cremoris* strains, the agar diffusion method of Tramer and Fowler (1964) was followed with modifications. *M. luteus* and *Lactococcus* strains were used as the indicator organisms in this assay. Inoculum of *M. luteus* was prepared by emulsifying the growth from YGM slope cultures with 7ml of 0.85% saline solution and if necessary, diluted to give a transmission of 50%. YGM agar, used also as assay agar, was autoclaved, cooled to 45°C and mixed with 2% (v/v) of *M. luteus* cell suspension. 50 ml of the seeded YGM agar was poured into each large sterile 150x15 petri-plate (VWR Scientific Company, Inc., West Chester, PA) and allowed to solidify. Wells (7.5 mm diameter x 3 mm depth) were aseptically punched in the seeded agar using a sterile cork borer. Cell-free supernatants from overnight MRS broth cultures of *Leu. cremoris* (putative inhibitor producers) were collected by centrifugation at 6000 rpm for 10 min. Wells were filled with 50 µl of culture supernatant, or with 50 µl of whole cell material i.e. complete MRS broth culture. Inhibition was detected by a zone of clearing around the well observed after overnight incubation at 28°C. The size of inhibition zones was measured from the edge of the well in only one direction to the edge of the clear zone. Nisin producer strain, *Lc. lactis* ATCC 7962, grown overnight in M17G broth, was used as positive control in the described well-diffusion assay. When *Lactococcus* strains (*Lc. lactis* 1363, *Lc.lactis* ML3; *Lc.lactis* C2; *Lc.cremoris*

205 and *Lc. cremoris* 224) were used as indicator strains in well diffusion assays, 0.2% (v/v) of a overnight M17G broth cultures were used to inoculate 50 ml of LM17 agar (M17 with 0.5% lactose) equilibrated at 45°C. The rest of procedure was the same as already described with *M. luteus* indicator organism.

(f) Associative growth of lactococci, 1%, and *Leu. cremoris* strains, 3%, in 11% RSM supplemented with 0.2% citrate, at 28°C was analyzed by plating the samples at the beginning, after 24 and after 48 hr of incubation. Each of twenty *Leu. cremoris* strains was combined with *Lc. lactis* ML3 or with *Lc. cremoris* 205. Total cell number (CFU/ml) was determined on MRS agar, while MRS agar containing 30 μg/ml vancomycin was used to selectively inhibit the growth of lactococci and therefore allowed a direct enumeration of *Leu. cremoris* in mixed cultures. Serial dilutions of cultures were prepared according to procedures described in the *Compendium of Methods for Microbiological Examination of Foods* (1976). Viable cell numbers of leuconostocs and lactococci in pure and in mixed cultures were compared. However, the main criterion for compatibility of tested mixed cultures was based on a positive reaction for King's test (King,1948).

3.3 Results and Discussion

Strain screening

Sixty strains of dairy leuconostocs originating from different collections have been accumulated over the years in our culture collection. Based on the taxonomic criteria traditionally used to define *Leu. cremoris*, twenty strains were so classified, while other strains were classified as *Leu. lactis*, *Leu. mesenteroides*, *Leu. dextranicum*, and also a few as *Lc. diacetylactis*. Behavior of *Leu. cremoris* strains in the tests used for their identification is described in this section.

All strains identified as *Leu. cremoris* were Gram-positive, coccoid bacteria grouped in pairs and chains. They were characterized by the production of D(-) lactic acid and CO_2 from glucose, and lack of L-arginine dihydrolase and catalase activity. They did not produce dextran from sucrose and were resistant to 50µg/ml of vancomycin. Growth on LUSM selective medium was very poor after five days of incubation. Presence of selective compounds, such as vancomycin, tetracycline and sodium azide, in LUSM medium drastically affected number of colonies as well as their size. Slightly better growth was observed at 22°C compared with growth at 28°C. Two *Leu. cremoris* strains, Da3 and Da15, did not grow on LUSM medium at all.

Even though, *Leu. cremoris* strains are reported to have a distinctive carbohydrate fermentation pattern (Fig. 3.1) with only lactose, glucose, galactose, and N-acetylglucosamine being fermented (Garvie,1984), four collection strains showed a different profile: Strain 30 weakly fermented maltose, strains 1040 and Lfa6 did not ferment galactose, and strain 44-4 did not ferment lactose, but intensively fermented fructose and mannose. Furthermore, ATCC type strain 19254 was unable to utilize lactose, but able to weakly ferment sucrose as also observed by Milliere et al.(1989). Although maltose and sucrose fermentation is not uncommon for *Leu. cremoris* (Whittenbury,1966), lactose and galactose defective strains most likely represent mutants of the parent strains.

Leu. cremoris strains did not grow at 37 or 45°C. None of the *Leu. cremoris* strains gave citrate-positive blue colonies on differential Kempler & McKay medium (Fig. 3.2), or on modified PMN agar. These media, although supplemented with yeast extract, were not suitable for leuconostocs since most of them grew poorly and failed to indicate citrate utilization. However, some strains of *Leu. lactis* and *Leu. dextranicum* gave blue colonies, indicating citrate utilization.

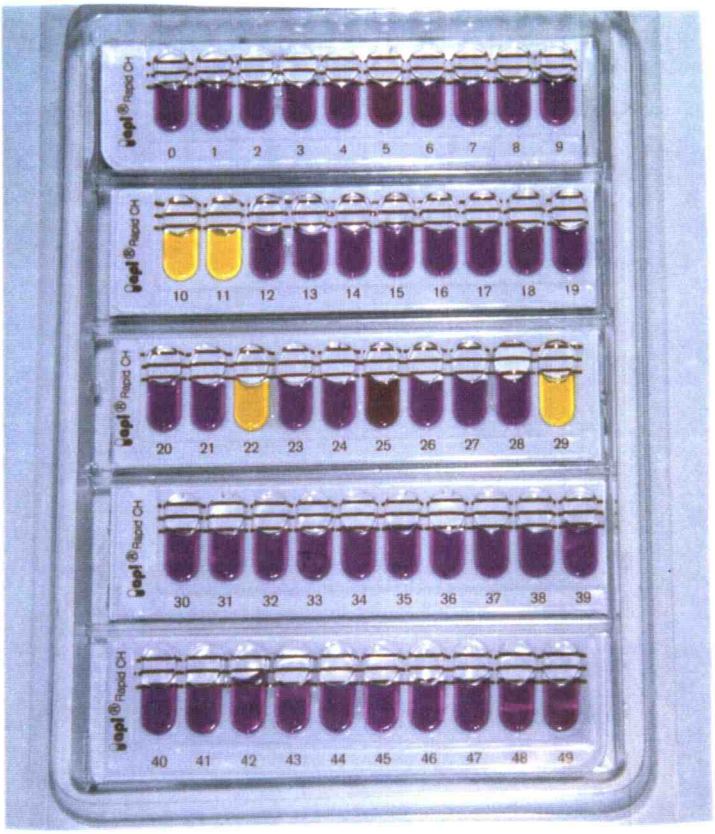

Figure 3.1 API card presenting the carbohydrate fermentation for *Leu . cremoris* L-5-319.

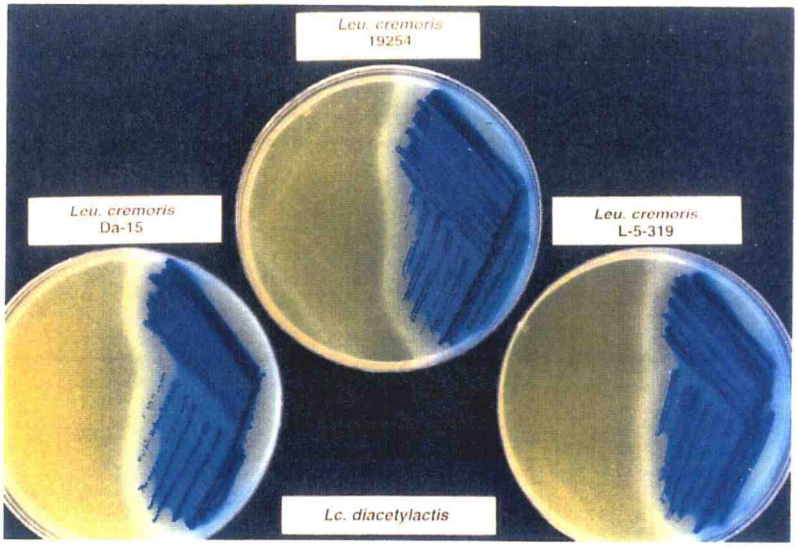

Figure 3.2 Growth of *Leu. cremoris* strains (left side of the petri-plates), and *Lc. diacetylactis* EmI (right side of the petri-plates) on citrate indicator agar.

Characteristics of *Leuconostoc mesenteroides* ssp. *cremoris* strains

(a) Growth and citrate utilization in milk.

None of the *Leu. cremoris* strains grew rapidly in milk according to spectrophotometrical analysis of milk cultures during incubation at 22 and 28°C. Although the method of Kanasaki et al. (1975) was not sufficiently sensitive, due to poor growth of leuconostocs in milk, growth patterns of different strains was possible to compare (Fig. 3.3). Strains 1039, 1040, 1041, and 30 grew better but slower at 22°C, while strains Da3, Da15, P1, A18, J, 122-5, and 173 produced higher populations at 28°C than at 22°C. Strains CAF7, 91404, 104, 176, L-5-319, CAE, Sepa1 and Lfa6 revealed similarity in growth at both temperatures.

Litmus milk was not changed or was only weakly acidified, pH 5.2 - 6.2, by the growth of *Leu. cremoris*. Acid production by *Leu. cremoris* was not affected by adding glucose or sodium citrate in 11% RSM (Table 3.2). Acid production was elevated, pH 4.4 -5.3, by enrichment of milk with 0.3% yeast extract, except for the lactose negative strains 19254 and 44-4. Growth of pure *Leuconostoc* cultures in milk supplemented with citrate did not result in diacetyl or acetoin production.

Although low pH values, which favor acetoin production, (<5.3), were reached in milk supplemented with yeast extract, no diacetyl or acetoin were detected by King's test during growth of dairy leuconostocs.

Citrate utilization by *Leu. cremoris* strains during growth in milk supplemented with citrate, measured by the sensitive enzymatic test, revealed some interesting results. Sixteen strains utilized citrate under neutral conditions, initial pH 6.5. Addition of yeast extract to the milk stimulated acid production and citrate utilization. According to Cogan et al. (1981) net acetoin production by *Leu. lactis* in the complete medium is a reflection of the competition between the inhibiting effect of glucose and the promoting effects of the medium constituents and the decrease in pH.

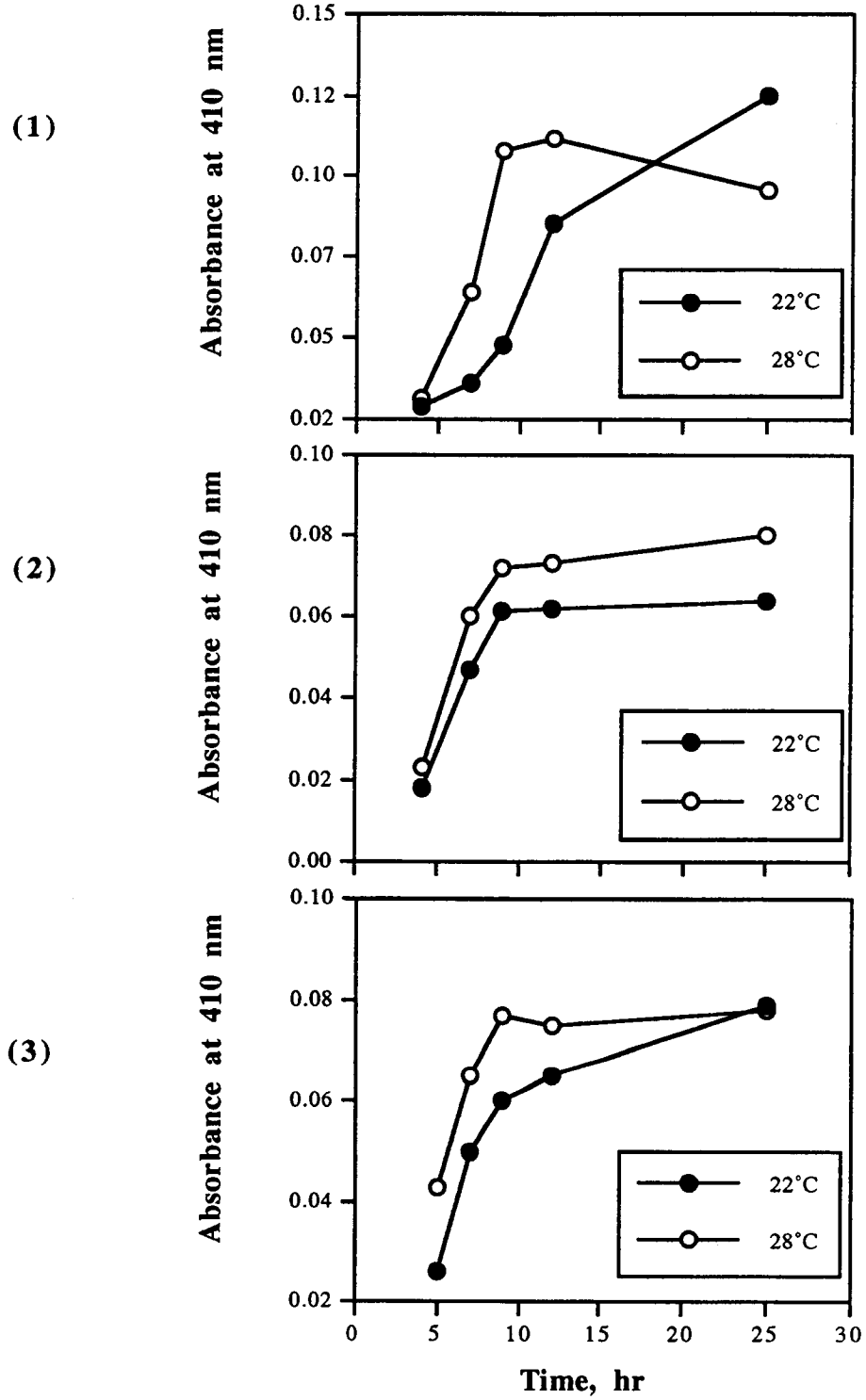

Figure 3.3 Spectrophotometric analyses of *Leu. cremoris* growth in milk at 22 and 28°C: (1) strain 1041; (2) strain P1; (3) strain 104.

Table 3.2 Acidity (pH) of *Leu. cremoris* milk cultures after 24 hr incubation at 28°C.

Strain	pH in 11% RSM	pH in 11% RSM + 0.2% citrate	pH in 11% RSM + 0.5% glucose	pH in 11% RSM + 0.3% yeast ext.
1039	5.2	5.5	5.5	4.4
1040	6.1	6.4	6.0	4.6
1041	5.7	5.7	5.7	4.5
Lfa6	5.9	5.8	5.5	4.8
Sepa1	5.7	5.9	5.8	5.3
P1	5.8	5.8	5.7	4.7
CAF7	5.8	5.9	5.9	4.6
91404	5.8	5.9	5.8	4.8
173	5.8	5.9	5.8	4.5
176	5.8	5.9	5.9	4.6
J	5.7	5.9	5.8	4.7
A18	5.8	5.9	5.8	4.6
104	5.8	6.0	5.8	4.6
CAE	5.6	5.8	5.5	4.8
Da3	5.9	5.8	5.9	4.6
Da15	5.9	5.7	5.9	4.5
L-5-319	5.6	5.9	5.6	4.4
122-5	5.4	5.6	5.5	4.4
30	5.7	5.9	5.8	4.8
44-4	6.2	6.4	5.6	6.2

In our experiments with *Leu. cremoris* citrate utilization was stimulated by yeast extract, most likely by generation of acidic conditions. In the presence of yeast extract, citrate was utilized in 9 hours, compared with 24-36 hours when yeast extract was not added to milk (Fig 3.4). However, no detectable amounts of diacetyl or acetoin were produced by these cultures, even though citrate was catabolized. These results are in agreement with Cogan's earlier results (Cogan,1975) which showed that yeast extract stimulated growth and citrate utilization by *Leu. cremoris* without concomitant production of diacetyl and acetoin. There is an explanation for this phenomena. In presence of lactose/glucose, the pyruvate produced from sugars and from citrate need to be reduced, e.g. to D-lactate, to

regenerate NAD. Lactate dehydrogenase (LDH) activity plays the major role in channeling pyruvate. Lack of an effect of pH on the apparent K_m of LDH for pyruvate in tested strains is a possible explanation for the absence of acetoin or diacetyl production at low pH (Cogan et al.,1981). Also, the utilization of citrate without production of diacetyl/acetoin may be explained by the inhibition of enzymes involved in citrate metabolism, such as acetolactate synthetase and decarboxylase, by some metabolites of glucose metabolism (Cogan et al.,1981; Cogan,1984). Moreover, according to Harvey and Collins (1963), and Schmitt et al.(1992), citrate could serve as a carbon source for the synthesis of some essential cell constituents, primarily lipids. No citrate metabolism occurred with strains 1039, 1040, 1041 and 44-4 (Fig. 3.5). Absence of lactose fermentation in strain 44-4 explains its inability to metabolize citrate which can not be used as an energy source by itself. It seems reasonable to assume that strains 1039, 1040, and 1041 have lost the plasmid-encoded citrate permease gene considered essential for citrate metabolism (Lin,1991).

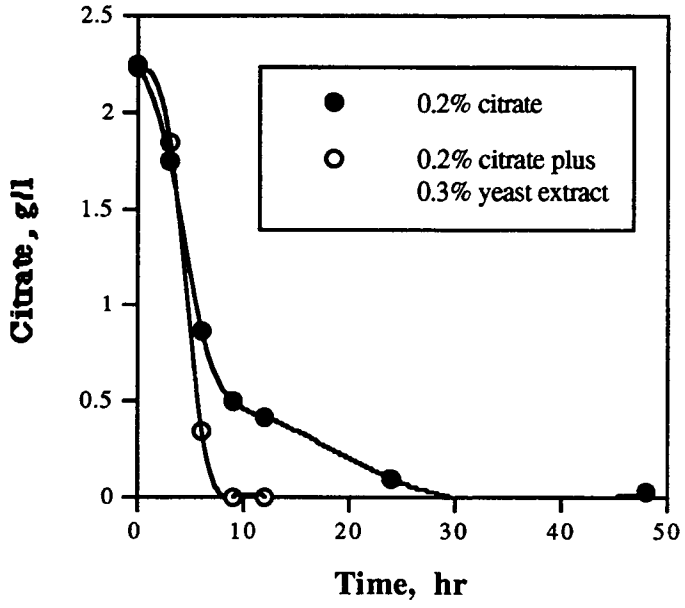

Figure 3.4 Citrate utilization by *Leu. cremoris* L-5-319 in milk supplemented with sodium citrate and yeast extract.

Negligible citrate uptake in presence of yeast extract may be attributed to diffusion of the uncharged, acidic form of citrate through the bacterial membrane at low pH, which is in agreement with the observation of Hugenholtz (1993).

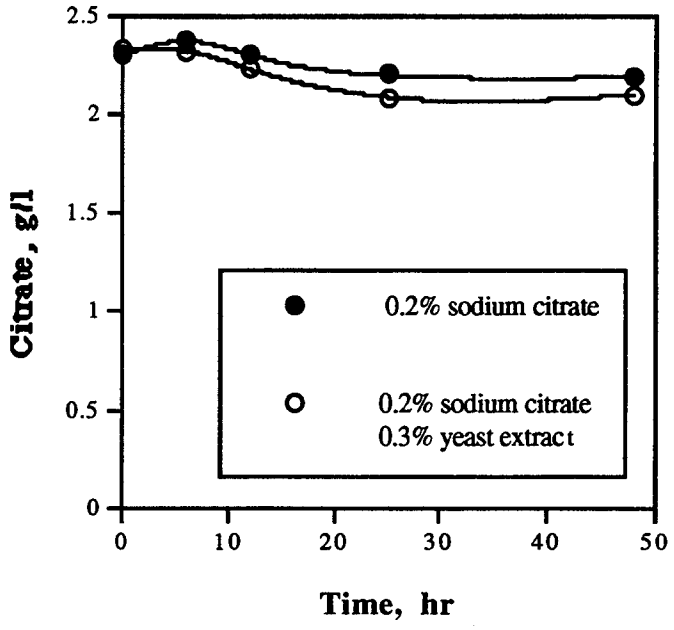

Figure 3.5 Citrate utilization by *Leu. cremoris* 1041 in milk supplemented with sodium citrate and yeast extract.

Preincubation of citrate positive strains for 24 hr in milk supplemented with citrate before addition of citric acid resulted in considerable production of diacetyl and acetoin (Chapter 4, Table 4.1). The addition of citric acid lowered the initial pH values of milk cultures to 4.3. These cultures were examined 18 hr after citric acid was added. Under these conditions, all cultures, except 1039, 1040, 1041 (cit-), and 44-4 (lac-), gave positive results in the King's test. Preincubation permitted leuconostocs to lower the carbohydrate content in milk, to utilize citrate, and, therefore, to reach sufficient cell densities to carry through additional citrate metabolism. With the addition of citric acid, extreme acidic conditions which favor acetoin/diacetyl production were attained, while active growth of the

cultures was suppressed, and precursor for diacetyl and acetoin production was supplied to the cultures. This way the production of aromatic compounds was favored, rather than synthesis of other metabolites and cell constituents from citrate.

(b) Diacetyl reductase (DR) activity

Lack of diacetyl accumulation in dairy products may be caused by destruction of diacetyl. Diacetyl reductase (EC 1.1.1.5) is the enzyme responsible for irreversible reduction of diacetyl to acetoin and further reduction to 2,3-butanediol, and is widely distributed among bacteria (Seitz et al.,1963). *Leu. cremoris* strains were compared for DR activities, and the results are presented in Table 3.3. Large differences in DR specific activity, ranging from 0 to 1603 U/mg, were observed between strains. Selection of strains low in DR for designing flavor-producing multiple-strain starter cultures is essential. In all strains that showed DR activity, the enzymes were NADH dependent, except in strain 44-4. Figure 3.6. represents spectrophotometric data typical for strains with high DR activity, like strain Da15, and for strains with low DR activity under experimental conditions, such is strain L-5-319. In *Leu.cremoris* 44-4, DR was active with either NADH or NADPH, but showed greater activity when NADH was used (Fig. 3.7). NADH dependent DR specific activity in strain 44-4 was 946 U/mg compared to 272 U/mg of NADPH dependent DR specific activity. One needs to be careful in interpreting results obtained for DR activity, because of a wide variety of enzymes that reduce keto groups and could occasionally interfere with diacetyl reductase assays in crude extracts. For example, Juni and Heym (1957) demonstrated that diacetyl is accepted, although poorly, as substrate by some alcohol dehydrogenases. Beside DR activity, it was also possible to follow NADH oxidase activity in cell-free extracts. NADH oxidation, in absence of diacetyl in reaction mixtures, was attributed to NADH oxidase activity. It was present, with great variability, in all cell-free extracts (Table 3.3), except in the strain CAE. Collins (1972) proposed three mechanisms by which the operation of NADH oxidase would boost diacetyl concentration in cultures.

All these mechanisms relate to the steps in carbohydrate metabolism that involve the regeneration of co-factor for recycling. The operation of NADH oxidase is thus important for regenerating NAD and its high activity may result in increased level of pyruvate available for diacetyl production.

Table 3.3 Diacetyl reductase and NADH oxidase activities in crude cell-free extracts of *Leu. cremoris* strains.

Strain	Specific DR activity[1]	Spec.NADH Oxidase activity[1]	Strain	Specific DR activity	Spec.NADH Oxidase activity
1039	43	107	L-5-319	0	252
1040	0	24	122-5	4	15
1041	86	150	173	0	8
Lfa6	1603	145	176	53	174
Sepa1	0	47	J	5	170
P1	0	4	A18	0	17
CAF7	0	19	CAE	0	0
91404	0	7	Da3	516	32
44-4	946	884	Da15	837	187
30	9	12	104	0	18
			19254	0	11

1) Specific activities are expressed in U/mg

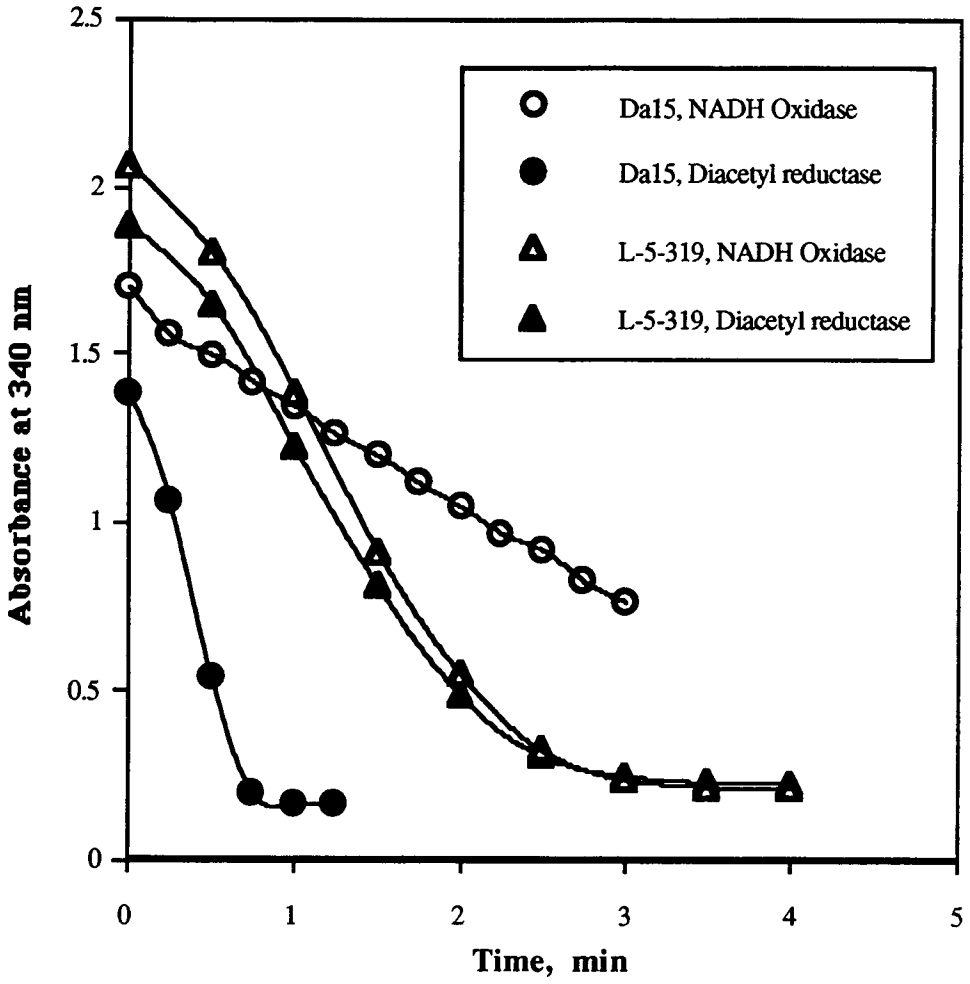

Figure 3.6 Oxidation of NADH in cell-free extracts of *Leu. cremoris* Da15 and *Leu. cremoris* L-5-319.

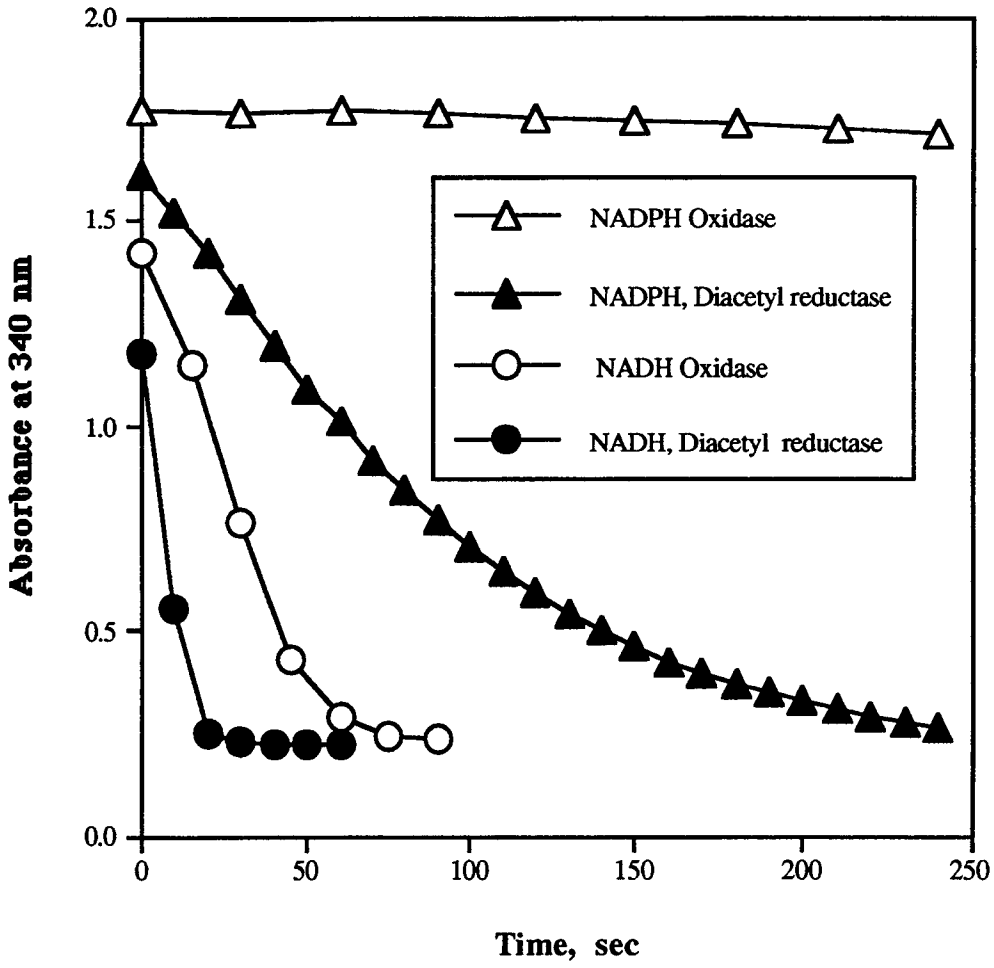

Figure 3.7 Oxidation of NADH and NADPH in cell-free extract of *Leu. cremoris* 44-4.

(c) Plasmid profiles

Since plasmid profiling has proven to be useful as a tool to differentiate *Leuconostoc* strains (Johansen and Kibenich,1992; Cavin et al.,1988), it was of interest to compare the plasmid profiles of the different *Leu. cremoris* strains. Only the sharp, bright bands were recorded, and approximate molecular weight of plasmids was determined by comparison with size standards included on each gel. Number and sizes of the plasmids varied greatly within *Leu. cremoris* strains (Fig. 3.8). All 20 strains examined contained from 1 to 5 plasmids with molecular weight values ranging from 4.4 kb to 55 kb. However,

seven strains: 91404, 173, 176, CAF7, 104, A18, and J showed very similar plasmid profiles (53.7, 26.1, 20 kb). Also, strains Da3 and Da15 were identical in plasmid content (15.3, 16.6, 18.0, 26.3, 45.5 kb). Strains with the same plasmid profiles may actually be identical, having come into the OSU culture collection from different sources but with different strain numbers. This emphasizes the need to be able to distinguish between strains that really are different and probably would involve DNA sequencing. Despite the similarity in plasmid profiles, some of the strains were still dissimilar in biochemical characteristics. In order to learn more about strain variability, further work also could be done on restriction enzyme analyses of *Leu. cremoris* chromosomal DNA.

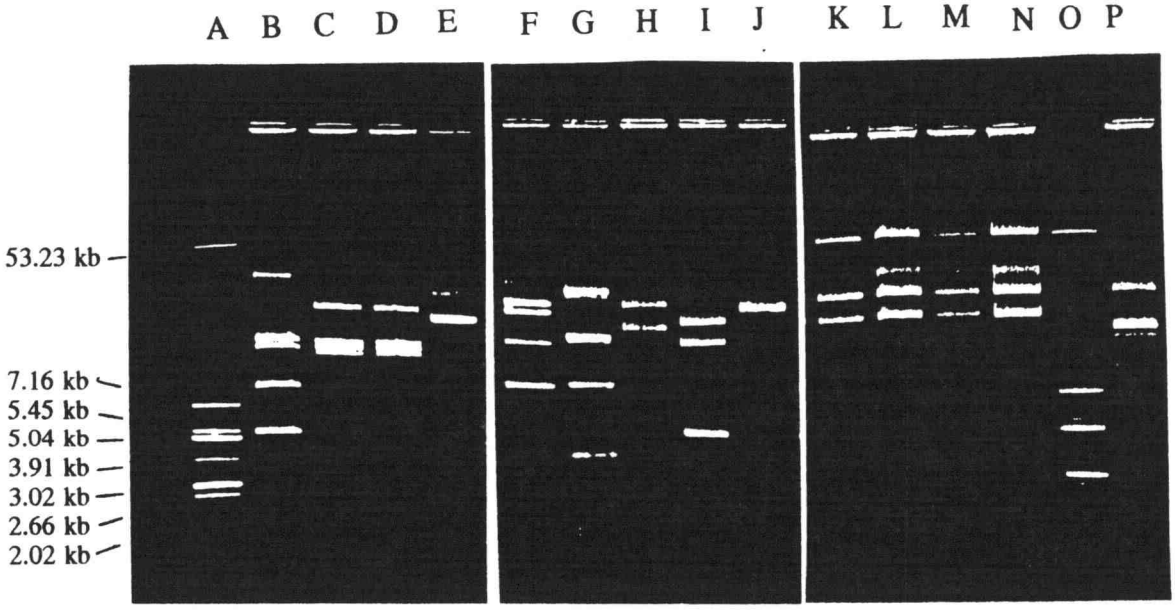

Figure 3.8 Plasmid profiles of *Leu. cremoris* strains. Lane A, size standard: *E.coli* V517; lane B, strain 19254; lane C, strain Da3; lane D, strain Da15; lane E, strain 44-4; lane F, strain 1040; lane G, strain 1041; lane H, strain CAF7, lane I, strain 122-5; lane J, strain CAE; lane K, strain A18; lane L, strain 104, lane M, strain 91404; lane N, strain J; lane O, *E.coli* V517; lane P, strain 30.

(d) Protein profiles

The overall cell soluble protein patterns of *Leu. cremoris* strains were very similar (Fig.3.9, 3.10 and 3.11). Fig.3.9 presents the protein pattern of strains with the same plasmid profiles (Strains: A18, 104, 91404, J, CAF 7, 173, 176). Although resolution of the low molecular weight proteins was low on this gel, due to a low concentration of bis-acrylamide (10 %), it was possible to notice a high degree of resemblance of their protein profiles. The concentration of separating gels for SDS-PAGE showed on Fig. 3.10 and 3.11 was increased from 10 to 12% to accomplish better separation of low molecular weight proteins. Protein electrophoregrams of all *Leu. cremoris* strains were similar. However, the protein gel of *Lc. diacetylactis* EmI showed additional bands, as well as the absence of some bands when compared with *Leu. cremoris* protein gels (Fig. 3.11).

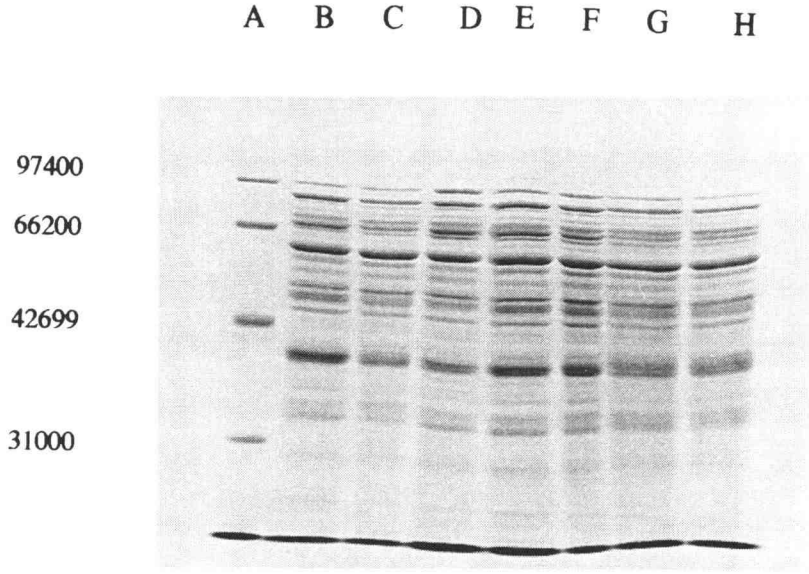

Figure 3.9 Protein profiles of *Leu.cremoris* strains. Lane A, molecular weight standard; lane B, strain A18; lane C, strain 104; lane D, strain 91404; lane E, strain J; lane F, strain CAF7; lane G, strain 173; lane H, strain 176.

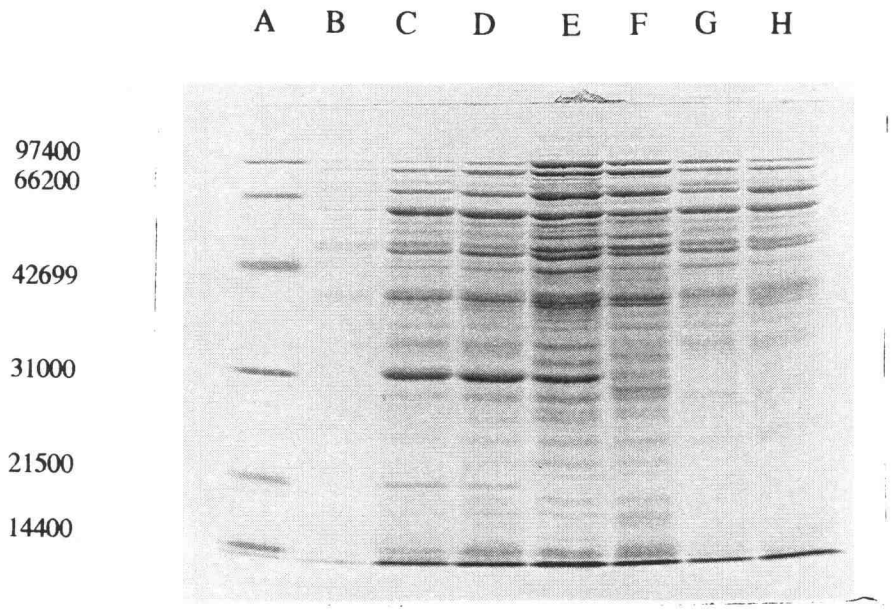

Figure 3.10 Protein profiles of *Leu.cremoris* strains. Lane A, molecular weight standard; lane B, strain 19254; lane C, strain Da3; lane D, strain Da15; lane E, strain Lfa6; lane F, strain 44-4; lane G, strain 122-5; lane H, strain 91404.

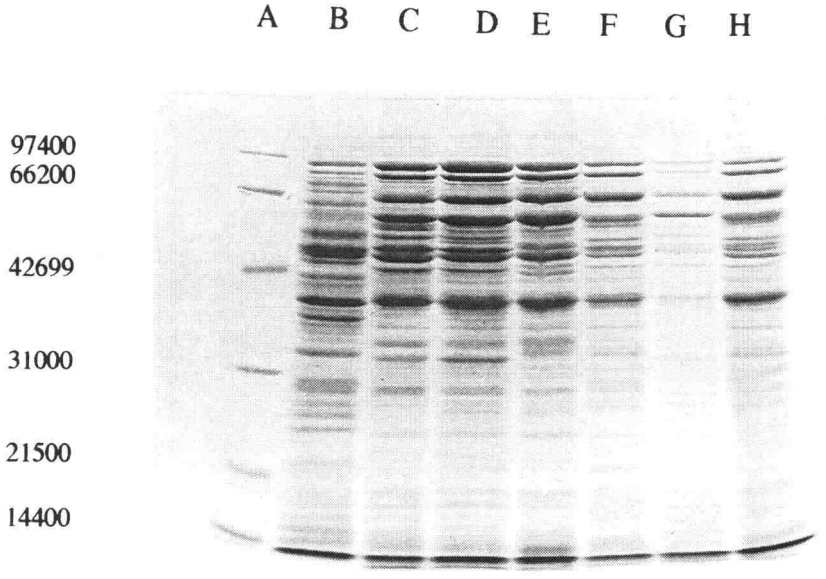

Figure 3.11 Protein profiles of *Leu. cremoris* strains *versus Lc. diacetylactis* strain EmI. Lane A, molecular weight standard; lane B, *Lc. diacetylactis* EmI; lane C, *Leu. cremoris* strain 30; lane D, strain Sepa1; lane E, strain CAE; lane F, strain 1039; lane G, strain 1040; lane H, strain 1041.

(e) Production of antimicrobial substances

Numerous workers have reported on the ability of *Leuconostoc* spp. to produce antimicrobial substances which are active against certain pathogenic and spoilage organisms (Sorrells and Speck,1970; Branen et al.,1975; Orberg and Sandine,1984; Hechard et al.,1992; Stiles,1994). Antimicrobial action of leuconostocs was generally attributed to organic acids, hydrogen peroxide, diacetyl and bacteriocins. The production of inhibitory substances by *Leuconostoc* spp. strains must be taken into consideration when composing mixed and multiple strain starter cultures.

Screening of *Leu. cremoris* strains for antimicrobial activity revealed that none of twenty *Leu. cremoris* strains inhibited *Lactococcus* strains used as indicators in well-diffusion assays. However, definite zones of inhibition were observed when *M. luteus* was used as indicator, and these were caused by a number of *Leu. cremoris* strains (Fig. 3.12 and 3.13). Whole broth cultures (whole cell material) of all twenty *Leu. cremoris* strains showed inhibition of *M. luteus*, with clear zones 1 to 5 mm in size (Table 3.4); on the other hand, only cell-free supernatants of strains 1039, 1040, 1041 and 44-4 caused inhibition of *M. luteus* and then it was slight. These data demonstrated the importance of viable cells or cell associated material for antimicrobial activity of *Leu. cremoris* strains against *M. luteus*. Similar findings have been reported for antimicrobial activity of *Lc. diacetylactis* (Vedamuthu et al.,1966). The inhibition was not caused by low pH, as demonstrated by the absence of clearing zone when hydrochloric acid, pH 4.5 was added to wells cut in agar seeded with *M. luteus*. The purpose of this study was not to further explore the nature of these inhibitory substances, since there was no antimicrobial activity by *Leu. cremoris* strains against *Lc. lactis* or *Lc. cremoris*.

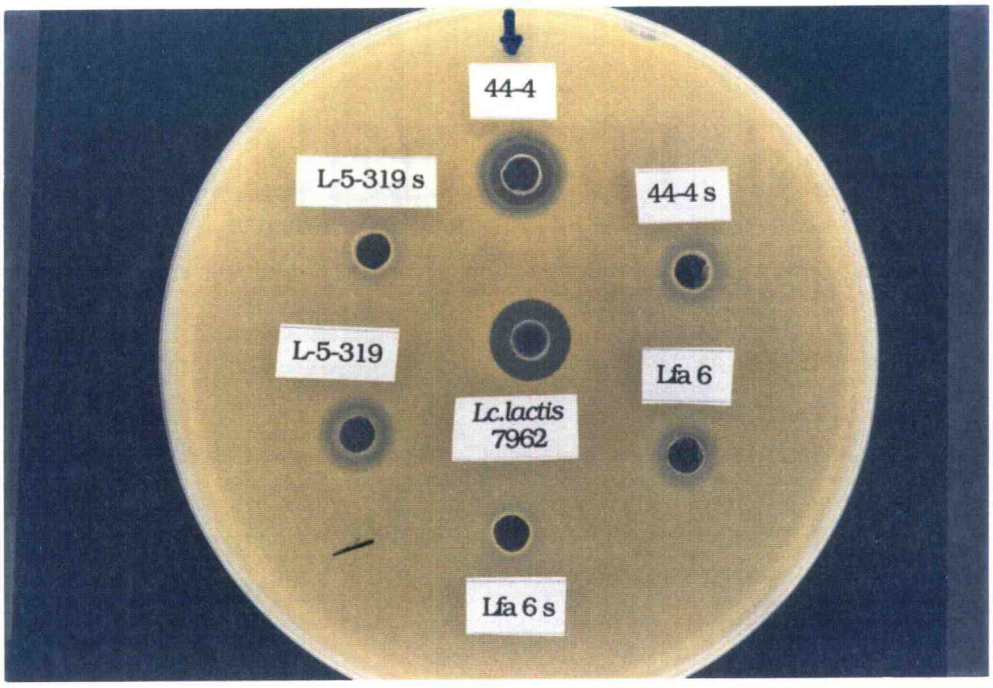

Figure 3.12 Well-diffusion inhibitory activity of whole MRS broth cultures and corresponding cell-free supernatants (S) of *Leu. cremoris* strains. Indicator organism is *M. luteus*, and positive control is *Lc. lactis* 7962.

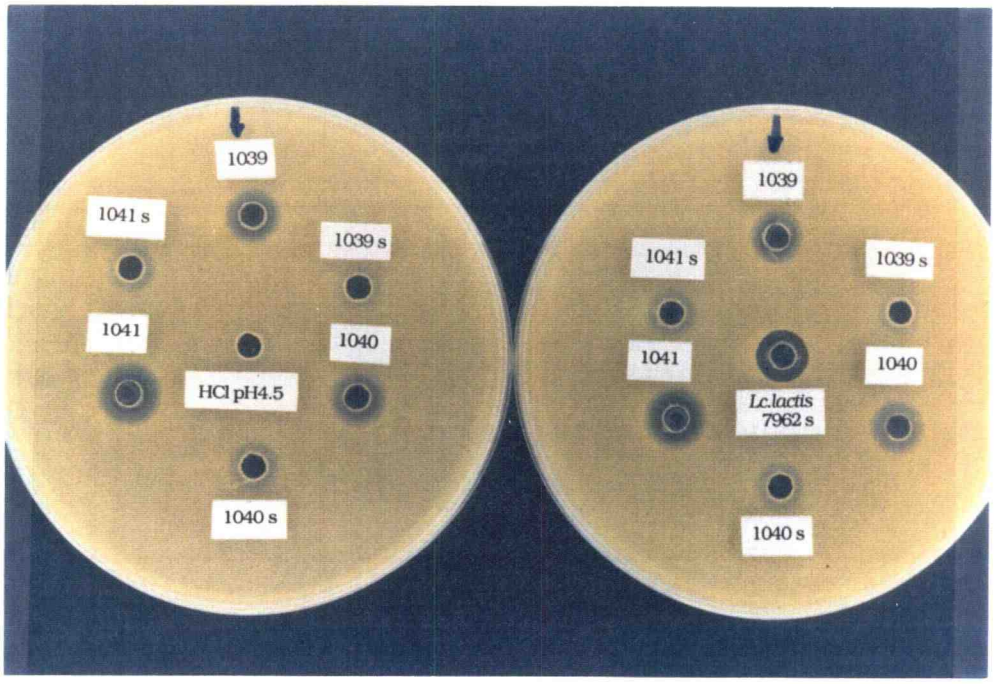

Figure 3.13 Well-diffusion inhibitory activity of whole MRS broth cultures and corresponding cell-free supernatants (S) of *Leu. cremoris* strains. Indicator organism is *M. luteus*, positive control is *Lc. lactis* 7962, and negative control is HCl solution, pH 4.5.

Table 3.4 Inhibition of *M. luteus* by whole MRS broth cultures of *Leu. cremoris* strains.

Strain	pH[1]	Zone of inhibition[2] [mm]	Strain	pH[1]	Zone of inhibition[2] [mm]
1039	4.4	4	L-5-319	4.6	3
1040	4.5	4	122-5	4.7	3
1041	4.4	5	173	4.7	2
Lfa6	4.5	3	176	4.8	2
Sepa1	4.6	3	J	4.8	1
P1	4.8	2	A18	4.8	1
CAF7	4.8	1	CAE	4.7	3
91404	4.6	2	Da3	4.5	3
44-4	4.3	5	Da15	4.5	3
30	4.5	3	104	4.9	1

1) *Leu. cremoris* strains were grown in MRS broth for 24 hr at 28°C
2) Each value is the average from three trials

(f) Associative growth and compatibility

Application of leuconostocs in dairy fermentations is based on associative relationships between these aroma-producers and acid-producing lactococci. This has been summarized by Vedamuthu (1994): "The associative growth relationship between these two groups of bacteria is symbiotic, and the functional relationship is synergistic". Screening the compatibility of *Leu. cremoris* strains with four *Lc. lactis* and six *Lc. cremoris* strains from our collection demonstrated that only strains 122-5 and 91404 gave positive King's test reactions when combined with both *Lactococcus* species. The reference strain used as a positive control in these King tests was *Lc. diacetylactis* 18-16. Also, production of diacetyl and acetoin by strains 91404 and 122-5 in combination with different *Lactococcus*

strains was not reproducible in all experimental replications. Unstable results here reflect the complexity of diacetyl production by leuconostocs in milk in the presence of lactococci, and emphasizes the complicated interrelationships between growth rate and citrate metabolism in mixed cultures.

In mixed cultures, *Leu. cremoris* reached 4 to 20% of the total population when grown in presence of *Lc. cremoris* after 24 hr of incubation at 28°C, based on the plate count. On the other hand, the proportion of *Leu. cremoris* in mixed cultures with *Lc. lactis* varied only from 2 to 12 % of the total population due to the final higher population of *Lc. lactis* compared to *Lc. cremoris.* (Tables 3.5 and 3.6). Using heavy inocula of leuconostocs, counts of 10^7 to 10^9 cells per milliliter of milk could be attained despite their poor growth in milk. Although it was reported that lactococci produce stimulatory substances necessary for the growth of leuconostocs (Holmes et al.,1968; Vedamuthu,1994), the cell counts of *Leu. cremoris* in pure and mixed cultures did not differ significantly. Strains such as P1, CAF7, 176, A18, reached nearly an equal number of cells in both mixed cultures, while strains such as 91404, 30 and L-5-319 attained somewhat higher populations in mixed cultures with *Lc. cremoris* than with *Lc. lactis.*

Table 3.5 Viable cell numbers of *Leu.cremoris* and *Lc.cremoris* in single and mixed-strain cultures after 24 hr incubation in milk at 28°C, determined by parallel plating on MRS agar and MRS agar containing 30µg/ml vancomycin.

Leu. cremoris strains	Single-strain culture of *Leu. cremoris* (CFU/ml x 10^6)		*Leu. cremoris* in mixed culture with *Lc. cremoris*[1] (CFU/ml x 10^6)		*Lc. cremoris*[1] in mixed culture with *Leu.cremoris* (CFU/ml x 10^6)	
	0 h	24 h	0 h	24 h	0 h	24 h
1039	92	730	44	250	14	1270
1040	22	81	20	86	19	2200
1041	31	156	34	380	10	1700
Lfa6	16	120	14	134	14	600
Sepa1	18	310	14	50	15	1400
P1	3	85	3	172	19	2500
CAF7	12	290	14	240	42	1000
91404	22	280	19	240	23	950
44-4	60	90	77	60	33	1220
30	13	85	8	156	24	1240
L-5-319	42	300	25	235	7	1060
122-5	9	260	6	110	18	1380
176	8	120	8	320	32	1780
J	6	144	8	260	32	1740
A18	6	190	6	216	32	900
CAE	20	70	22	190	35	1440
Da3	34	133	41	100	37	1550
Da15	27	350	56	198	35	1330
104	7	164	5	250	15	-

1) *Lc. cremoris* 205

Each value is the average from three trials

Table 3.6 Viable cell numbers of *Leu.cremoris* and *Lc.lactis* in single and mixed-strain cultures after 24 h incubation in milk, at 28°C, determined by parallel plating on MRS agar and MRS agar containing 30µg/ml vancomycin.

Leu. cremoris strains	Single strain culture of *Leu. cremoris* (CFU/ml x 10^6)		*Leu. cremoris* in mixed culture with *Lc. lactis*[1] (CFU/ml x 10^6)		*Lc. lactis*[1] in mixed culture with *Leu.cremoris* (CFU/ml x 10^6)	
	0 h	24 h	0 h	24 h	0 h	24 h
1039	92	730	53	220	40	2830
1040	22	81	17	90	63	3410
1041	31	156	38	360	25	2660
Lfa6	16	120	24	176	66	1820
Sepa1	18	310	18	105	69	2800
P1	3	85	3	130	41	1520
CAF7	12	290	15	290	92	2110
91404	22	280	18	120	56	2800
44-4	60	90	76	90	68	3400
30	13	85	8	27	42	2470
L-5-319	42	300	26	130	44	-
122-5	9	260	13	116	63	3460
176	8	120	7	280	67	2720
J	6	144	6	320	84	-
A18	6	190	4	190	54	2510
CAE	20	70	16	220	34	1880
Da3	34	133	24	96	41	4200
Da15	27	350	42	172	54	3680
104	7	164	10	193	65	2800

1) *Lc. lactis* ML3
Each value is the average from three trials.

3.4 Conclusions

1. Among sixty strains of leuconostocs originating from different sources, twenty strains were classified as *Leuconostoc mesenteroides* ssp. *cremoris*;

2. Absence of lactose fermentation was observed with strain 44-4 and with ATCC reference *Leu. cremoris* strain 19254;

3. Three *Leu. cremoris* strains (1039, 1040, 1041) did not utilize citrate in milk;

4. Citrate positive *Leu. cremoris* strains utilized citrate in milk much faster in the presence of yeast extract with no concomitant production of diacetyl or acetoin;

5. Addition of citric acid to *Leu. cremoris* milk cultures after a preincubation period stimulated diacetyl and acetoin production during additional incubation period;

6. In mixed culture studies, only two *Leu. cremoris* strains (91404 and 122-5) were able on occasion to produce amounts of diacetyl and acetoin detectable by the King's test;

7. *Leu. cremoris* strains revealed large differences in specific activity of diacetyl reductase ;

8. All *Leu. cremoris* strains contained from 1 to 5 plasmids with molecular weight values ranging from 4.4 kb to 55 kb. Seven strains showed identical plasmid profiles;

9. Overall cell soluble protein patterns of *Leu. cremoris* strains were very similar;

10. Inhibition of *M. luteus* by viable *Leu. cremoris* cells was evident in well-diffusion plate assays. None of twenty *Leu. cremoris* strains inhibited *Lactococcus* strains used as indicators in applied well-diffusion assays;

11. *Leu. cremoris* 91404 was selected for further studies of milk fermentation in order to produce flavorful buttermilk.

CHAPTER 4

Use of *Leuconostoc mesenteroides* ssp. *cremoris* 91404 to improve milk fermentations

4.1 Introduction

A major defect in cultured dairy products is lack of well-balanced flavor (Vasavada and White,1979; Vedamuthu,1985; Lundstedt and Corbin,1983). Although diacetyl is the key flavor compound in cultured dairy products, other volatile components such as acetaldehyde, ethanol and acetic acid, contribute to the totality of the flavor (Keenan and Bills,1968; Belin et al.,1992; Linton and Wright,1993). Based on examination of several mixed-strain butter cultures for diacetyl and acetaldehyde content, Lindsay et al. (1965) proposed that for a desirable, balanced flavor in butter cultures, the diacetyl-to-acetaldehyde ratio should be between 4.4:1 to 3.2:1. Carbon dioxide produced by starter cultures, provides the effervescence and the "lift" to cultured buttermilk very similar to its role in carbonated beverages (Vedamuthu,1994).

Many methods for detection and quantification of volatiles, particularly diacetyl and acetoin, have been developed over the years (Westerfeld,1945; Pack et al.,1964; Walsh and Cogan,1974). Numerous investigators have used gas-liquid chromatography (GLC) and HPLC to measure the fermentation products of bacterial metabolism (Jansen et al.,1979; Bednarski et al.,1989; Ulberth, 1991; Starrenburg and Hugenholtz,1991), although those methods usually suffered from the disadvantage that pretreatment of samples, with an acidification-extraction procedure followed in many cases by a derivatization procedure, was necessary. Simple and direct chromatographic analyses of the end products of lactic acid bacteria without an extraction process (Palo and Ilkova,1970; Thornhill and Cogan,1984; Yamada,1989) are most useful. In the present study, direct injection of cultures on a GLC column and subsequent detection of volatile compounds produced in milk fermentation, with special attention to diacetyl and acetoin, was applied. The GLC

method described in this chapter was found to be a simple and exact method to analyze volatile end products of *Leuconostoc* metabolism.

The goal of this study was to improve the flavor and consistency of cultured buttermilk. Among the various biological and environmental factors that influence the development of buttermilk flavor (Frank,1984), we have mainly concentrated on selection of starter cultures and the relationship existing between citrate utilization and diacetyl and acetoin production during fermentation. Improvement of milk fermentations by using *Leu. cremoris* 91404 as the flavor producer, and *Lc. cremoris* as the acid producer in multiple starter cultures, was investigated and reported in this chapter. Use of *Leu. cremoris* 91404 in manufacture of buttermilk was based on its satisfactory characteristics such as low diacetyl reductase activity, high diacetyl production from citrate under acidic conditions, and compatibility with acid-producing lactococci.

4.2 Material and Methods

Organisms and growth conditions

Leuconostoc mesenteroides ssp. *cremoris* (*Leu. cremoris*) strains 91404 and 122-5, and *Lactococcus lactis* ssp. *cremoris* (*Lc. cremoris*) strains 205 and 352 used in this study were from the culture collection of the Dairy Microbiology Laboratory at Oregon State University. *Leu. cremoris* 91404 was originally obtained from Moseley Laboratory, Indianapolis, IN, but also is available from the American Type Culture Collection, Rockville, MD. *Leu. cremoris* strains were grown in MRS broth (55g/l of dehydrated MRS broth; Difco, Detroit, MI) supplemented with 0.1% sodium citrate for 24 hr at 28°C, unless otherwise stated. Cultures were maintained in MRS broth at 4°C and frequently subcultured. All procedures pertaining to characterization of *Leu. cremoris* 91404 were the same as have been described in Materials and Methods of Chapter 3.

Identity of *Lc. cremoris* strains 205 and 352 was confirmed by application of a subspecies-specific rRNA probe for *Lc. cremoris* (68RCa) in the whole-cell dot blot hybridization procedure (Salama et al.,1991). *Lc cremoris* strains were propagated in 11% RSM for 18 hr at 22°C. Cultures were maintained in 11% RSM, at 4°C following inoculation without previous incubation, and later incubated overnight at 22°C as needed. Stock cultures of *Leu. cremoris* and *Lc. cremoris* were stored at - 70°C in sterile 11% RSM supplemented with 20% (v/v) glycerol.

Growth of *Leu. cremoris* in milk

Growth curves of *Leu. cremoris* 91404 in milk were determined as follows. Three percent of a *Leuconostoc* culture grown for 24 hr at 28°C in MRS broth supplemented with 0.1% sodium citrate was used to inoculate 500 ml of sterile 11% RSM supplemented with 0.2% sodium citrate as needed. One percent of a *Lc. cremoris* culture grown for 18 hr at 22°C in milk, along with 3% of a 91404 MRS culture, were used as the inoculum in mixed culture experiments. To define growth of *Leu. cremoris* 91404 in pure and in mixed cultures, 5 ml samples were pipetted from milk cultures at certain time intervals during incubation at 22°C, and serial dilutions prepared according to procedures described in the *Compendium of Methods for Microbiological Examination of Foods* (1976). Diluted samples were plated for viable cell counts on both MRS agar and MRS agar containing 30μg/ml vancomycin. All culture plates were incubated at 28°C for 24-36 hr. In mixed culture studies *Leuconostoc* counts was based on number of colonies (CFU/ml) grown on MRS agar supplemented with vancomycin, while the *Lactococcus* count was determined by subtracting the *Leuconostoc* count from the total viable cell count obtained on MRS agar.

Specific growth rates (k) were determined from the exponential phase of the growth curves (ln CFU/ml plotted versus time) as

$$k = \frac{\ln X_2 - \ln X_1}{t_2 - t_1}$$

where t_1 and t_2 are the times at which the corresponding colony numbers X_1 and X_2 were determined. Generation times (g) were calculated from k values using equation:

$$g = \frac{\ln 2}{k}$$

In the same way growth of *Leu. cremoris* 91404 was followed during preparation and storage of experimental buttermilk.

Mather and Babel method

Diacetyl and acetoin production by pure *Leu. cremoris* cultures under acidic conditions was tested using the Mather and Babel (1959) method modified as follows. *Leu. cremoris* strains were grown for 24 hr at 28°C in 11%RSM after which the pH was adjusted to pH 4.3 with 1M citric acid or with 2M lactic acid. Uninoculated 11%RSM treated in the same manner was used as a negative control. After incubation for an additional 18 hr, the amounts of diacetyl and acetoin in acidified milk cultures were measured by GLC.

Preparation of experimental buttermilk

Portions (500 ml) of lowfat (1% fat) milk purchased from Fred Meyer, Corvallis, OR, and supplemented as needed with 0.1% sodium citrate, were dispensed into glass milk bottles and pasteurized by steaming for 45 min. After cooling, milk was inoculated with *Leu. cremoris* 91404 (3%) and *Lc. cremoris* (1%) 205 or 352, and incubated at 22°C until pH 4.5-4.6 was reached (about 18 hr). After incubation, the buttermilk was gently shaken to break the curd and 0.1 to 0.15% sodium citrate or 0.15% citric acid was added from 30% or 15% stock solutions, respectively. Shaking was repeated after fortification with citrate or citric acid . The buttermilk was then stored at 5°C. During the ripening and storage period, 5 ml samples were taken at various time intervals and analyzed for viable cell counts, citrate and volatile compound content. Time was counted from the moment of inoculation (0 hr).

Citrate utilization by *Leu. cremoris* 91404 in milk was determined using the enzymatic analysis kit (Boehringer/Mannheim, GmbH). Concentrations of volatiles in the samples were determined by GLC analyses.

Hydrogen peroxide-catalase milk treatment

The effect of hydrogen-peroxide treatment of milk on diacetyl levels in milk cultures containing *Leu. cremoris* 91404 was examined using the method of Pack et al. (1968a).

Hydrogen peroxide, 30% solution, and bovine liver catalase (Sigma, St. Louis, MO) were used for treatment of sterile 11% RSM (121°C, 12 min) supplemented with 0.2% sodium citrate. Hydrogen peroxide (0.03%) was added and mixed into 100 ml of milk. After 15 min at room temperature, 0.001% of catalase was added to the mixture from a 0.1% stock solution, and allowed to react for 5 min. This amount of catalase was sufficient to completely decompose the hydrogen peroxide, as determined colorimetrically by the following procedure: 5 drops of freshly prepared 40% potassium iodide was added to 10 ml of milk treated with 0.03% hydrogen peroxide and different amounts of catalase

(0.0001-0.002%). Control milk not treated with hydrogen peroxide was prepared in the same manner. The appearance of a yellow discoloration, compared with the control tube, indicated the presence of hydrogen peroxide that caused oxidation of potassium iodide liberating free iodine to color the milk.

Hydrogen peroxide-catalase treated milk was inoculated with pure *Leu. cremoris* 91404 culture at a concentration of 3%, or with mixed culture containing 3% *Leu. cremoris* 91404 and 1% *Lc. cremoris* 205, and incubated at 22 or 30°C. Samples were collected and analyzed by GLC.

Agitated milk cultures

Lowfat milk (1%) pasteurized by steaming for 45 min was inoculated with 3% *Leu. cremoris* 91404 and 1% *Lc. cremoris* 205. Agitated fermentation was carried out in a 1L cylindrical bottle containing 500 ml of milk and incubated on a shaker, at 22°C for 24 hr. Agitation was performed by shaking at a speed of 250 rpm. Samples were analyzed by GLC.

Gas - liquid chromatographic analyses

The amounts of volatile compounds produced by *Leu. cremoris* in milk under experimental conditions were measured by GLC using a model 5170A, Hewlett Packard gas chromatograph equipped with a flame ionization detector and coupled with a 3390A, Hewlett Packard integrator. The glass column (Supelco, Bellefonte, PA), 2m long and 2mm in diameter, packed with 80/120 Carbopack B AW/6.6% Carbowax 20 M was suitable for direct determination of diacetyl, acetoin, ethanol, and acetaldehyde in the samples. N_2 was used as carrier gas at a flow rate of 20 ml/min at 60 psi. H_2 was used at 34 psi, and air at 26 psi. The column temperature was programmed to increase from 90 to 130°C at rate of

2°C/min, while temperatures of injection port and detector were set at 170 and 150°C, respectively.

Concentrations of volatiles were determined by the Internal Standard calculation for the integrator used. Sec-butanol was used as internal standard (IS) at a final concentration of 50 ppm. Standard solutions were prepared daily from stock solutions. Fresh stock solutions of standards (acetaldehyde, ethanol, diacetyl, acetoin and acetic acid) were prepared each week by dissolving the volatiles (Aldrich, Milwaukee, WI) in Millipore, deionized water to give 1000 ppm solutions. Fine tuning of GLC with fresh standards each day was part of the protocol for GLC analysis. Standard curves were plotted from fresh aqueous solutions of standards and were used to quantitate the amounts of volatiles in milk cultures. Concentration of each compound was calculated by comparing the ratios of compound : IS peak areas in the samples and standard solutions (Thornill and Cogan,1984). To investigate whether or not components of milk interfered with GLC analyses, peak areas from injection of aqueous standard solutions were compared with peak areas of milk-based standards. Milk-based standards were prepared by adding the known amount of volatile compounds to the supernatant of uninoculated milk acidified with lactic acid (60% v/v) to pH 4.5. After filtration through 0.45 μm acrodisc filter and dilution with 1 mM sec-butanol in 1:1 ratio, milk-based standards (0.5 μl) were injected into the GLC column.

Samples of milk cultures were prepared as follows. Samples were cooled on ice, then clarified by centrifugation at 13,000 rpm for 10 min and filtered through 0.45 μm-pore-size filter. Filtrates were diluted in 1:1 ratio with 1 mM sec-butanol, and 0.5 μl of this mixture was injected into the gas chromatograph.

4.3 Results and Discussion

Characteristics of *Leu. cremoris* 91404

Large strain differences observed throughout physiological and biochemical testing of *Leu. cremoris* strains presented in Chapter 3, indicated that some strains, even used commercially, are not suitable for production of good flavor in cultured dairy products. Selection of strains with satisfactory characteristics such as low diacetyl reductase (DR) activity, high diacetyl production from citrate under acidic conditions, and compatibility with acid-producing lactococci, is essential (Lundstedt,1983; Hugenholtz,1993; Vedamuthu,1994). Characterization of *Leu. cremoris* strains (Levata-Jovanovic and Sandine, 1994a) was the basis for selection of strain 91404 for further study of buttermilk fermentation. The following characteristics were determinative:

a) Low DR activity. No DR activity, indirectly measured by the rate of oxidation of NADH or NADPH, was found in cell-free extracts of 91404 under experimental conditions used (Fig.4.1).

b) The ability to utilize citrate under neutral (pH 6.5) and acidic conditions (pH 4.3). Nevertheless, strain 91404 did not produce diacetyl or acetoin in 11% RSM at initial neutral pH, even though citrate was catabolized. Addition of yeast extract to the milk stimulated citrate utilization without concomitant production of diacetyl or acetoin. Complete disappearance of citrate from milk supplemented with 0.3% yeast extract occurred in 9 h (Fig. 4.2) and was most likely stimulated by some components of yeast extract and by generation of acidic conditions. The results were the same at either 22 or 28°C. The lack of diacetyl and acetoin production by growing leuconostocs may be explained by channeling pyruvate formed from citrate to lactate as a means of oxidized NAD regeneration, by synthesis of essential cell constituents such as lipids from citrate (Schmitt et al.,1992), by inhibition of enzyme activity by some intermediates of sugar metabolism (Cogan et al.,1981), by the effect that variation in pH_{in} may have on rate of enzyme reactions within

cell (Fitzgerald et al.,1991), or by rapid decrease of redox potential during active growth (Monnet et al.,1994).

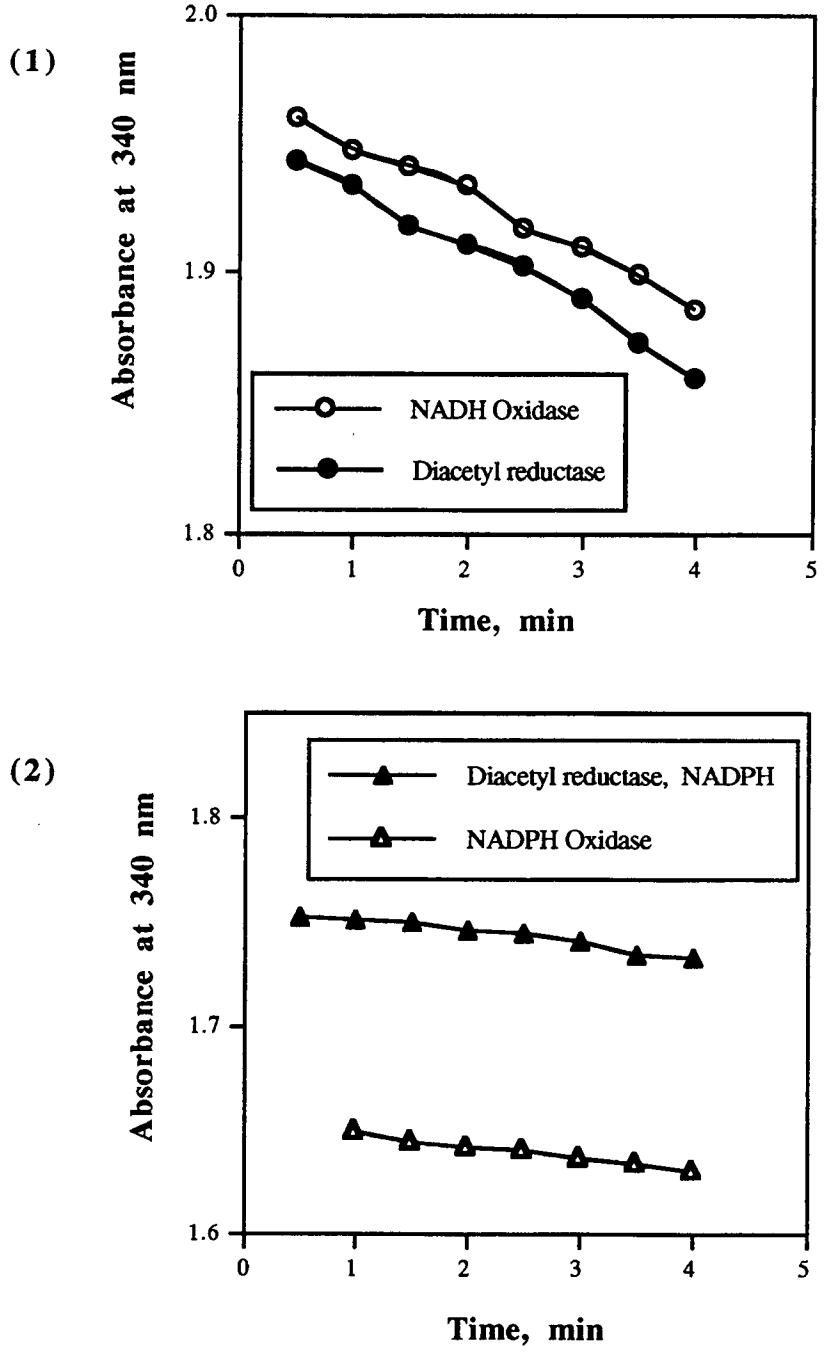

Figure 4.1 Oxidation of (1) NADH and (2) NADPH by diacetyl reductase and NADH oxidase in cell-free extract of *Leu. cremoris* 91404.

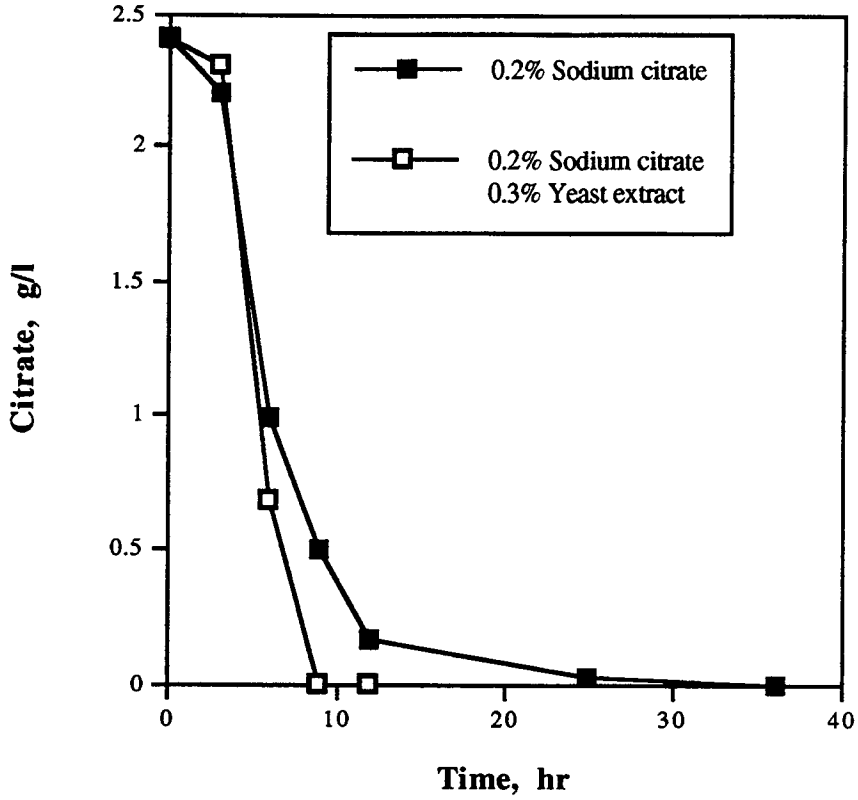

Figure 4.2 Citrate utilization by *Leu. cremoris* 91404 in milk supplemented with sodium citrate and yeast extract.

Importantly, high levels of diacetyl and acetoin, detected by the King's test and quantified by GLC, were produced by 80% of *Leu. cremoris* strains (Table 4.1) in preincubated cultures, additionally fortified with citric acid (Mather and Babel method,1959). Cessation of active growth, as the result of sugar exhaustion during preincubation period, and extreme acidic conditions created by addition of citric acid (pH 4.3) favored diacetyl/acetoin production during the additional incubation period. This is in agreement with the results of Cogan et al. (1981) related to the production of acetoin and diacetyl in acidified (non-growing) *Leuconostoc* cultures. Considering the ability of *Leu. cremoris* 91404 to utilize citrate under neutral conditions, it is reasonable to speculate that addition of citric acid, after sugar has been metabolized, provides necessary precursor for production of flavor compounds in non-growing *Leuconostoc* cultures, beside its role in

creating favorable acidic conditions. When the same experiment (Mather and Babel method) was conducted with 2M lactic acid instead of 1M citric acid, no diacetyl or acetoin were detected in tested cultures. These results are also in agreement with findings of Snoep et al. (1992) that at low pH and in the presence of citrate, the high internal accumulation of pyruvate favors the activity of α-acetolactate synthase, which has very low affinity for pyruvate.

Table 4.1 Concentrations of diacetyl and acetoin produced by *Leuconostoc mesenteroides* ssp. *cremoris* strains in the presence of citrate, at pH 4.3.

Strain	Initial pH[1]	Diacetyl[2] [ppm]	Acetoin[2] [ppm]
Lfa6	5.7	2.8	169.6
Sepa1	5.9	10.0	173.9
P1	5.8	70.6	472.3
91404	**5.9**	**75.3**	**326.6**
30	5.9	25.6	764.4
L-5-319	5.6	24.9	558.0
122-5	5.5	15.6	162.1
CAE	5.5	9.0	394.4
Da15	5.9	39.0	365.3

1) Cultures were preincubated in milk for 24 hr at 28°C before addition of citric acid.
2) Diacetyl and acetoin were determined by GLC after additional 18 hr incubation.

c) Growth characteristics in pure and mixed milk cultures. For functionality, associative culturing of lactococci and leuconostocs needs compatible strains and also sufficient numbers of the bacteria that produce acid and aroma. In comparison with literature data on growth rate and generation time of *Leu. cremoris* (Goel and Marth,1969; Cooper and

Collins, 1978), strain 91404 tended to have a shorter generation time during incubation in milk. Figure 4.3 illustrates the differences in growth pattern between two *Leu. cremoris* strains from our culture collection. Average generation time at 22°C, which is the temperature commonly used for buttermilk production, for strain 91404 was 86 min compared with 192 min for strain 122-5.

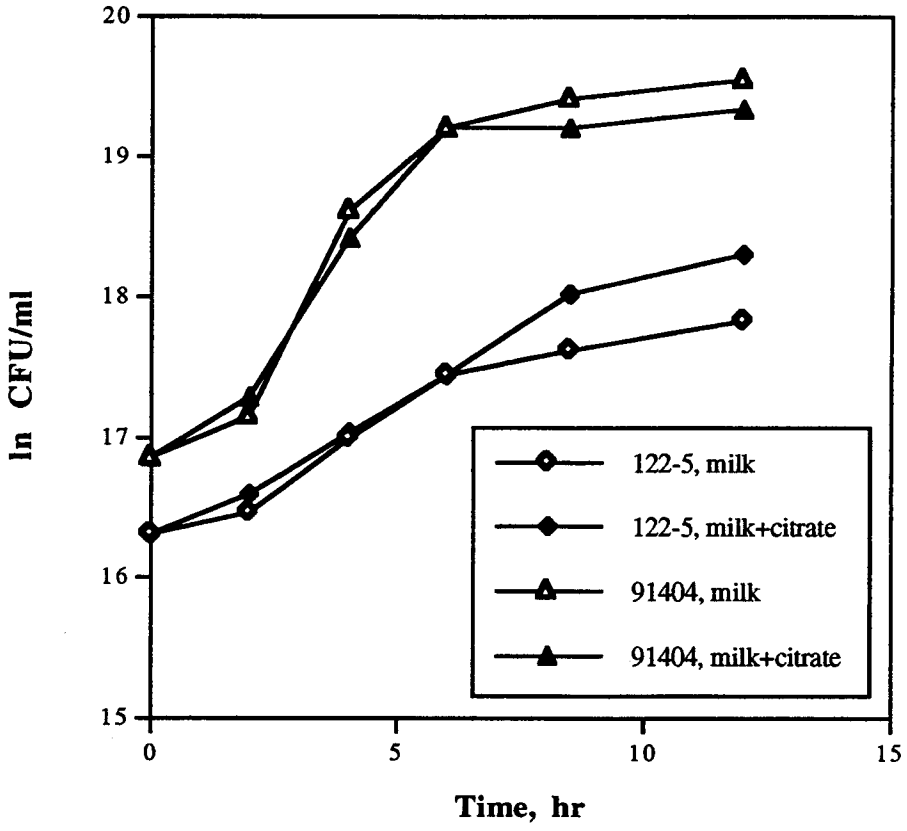

Figure 4.3 Growth of *Leu. cremoris* strains 91404 and 122-5 in milk at 22°C.

Examining the growth curves of *Leu. cremoris* 91404 (Fig. 4.4) revealed the similarity in growth throughout early incubation at 22 and 28°C, with some reduction during the exponential phase at 28°C. Generally, the pure culture attained scarcely a higher population at 22°C ($31x10^7$ CFU/ml) than at 28°C ($19x10^7$ CFU/ml) as measured after

12 hr of incubation by the plate count. The growth rate for the culture grown in 11% RSM fortified with citrate to 0.2% appeared to be very similar to the growth rate in 11% RSM (Fig. 4.4). Literature data on the effect of citrate on growth rates of leuconostocs vary. It was found that most strains of leuconostocs grew faster on sugar plus citrate than on sugar alone, explained by increased production of ATP through the activity of acetate kinase from processor acetyl-phosphate due to a switch from ethanol to acetate production (Cogan,1987; Starrenburg and Hugenholtz,1991). However, Drinan et al. (1976) found that the presence of citrate had no effect on the growth rate of the leuconostocs, although the final amount of acid produced was somewhat higher in the case of citrate-grown cells. However, it is difficult to compare our results with literature data, considering the different media that were used (MRS *versus* milk), different preparation of inocula, different methods used for monitoring growth, different strains, even species, that were studied and lack of results on growth of leuconostocs in milk completely void of citrate.

Balanced growth of lactococci and leuconostocs in milk at 22°C was observed in experiments with mixed starter cultures (Fig.4.5). The average generation time of *Leu. cremoris* 91404 in mixed culture (85 min) did not differ from the estimated generation time of the pure culture (86 min), indicating that growth of 91404 was not stimulated, nor inhibited by *Lc.cremoris* 205. The average generation time of the metabolically more active *Lc. cremoris* was 65 min. In cultured buttermilk production it is important to keep the incubation temperature between 21 and 25°C, because at temperatures above 25°C the ratio is skewed toward fast growing lactococci (Gilliland,1985).

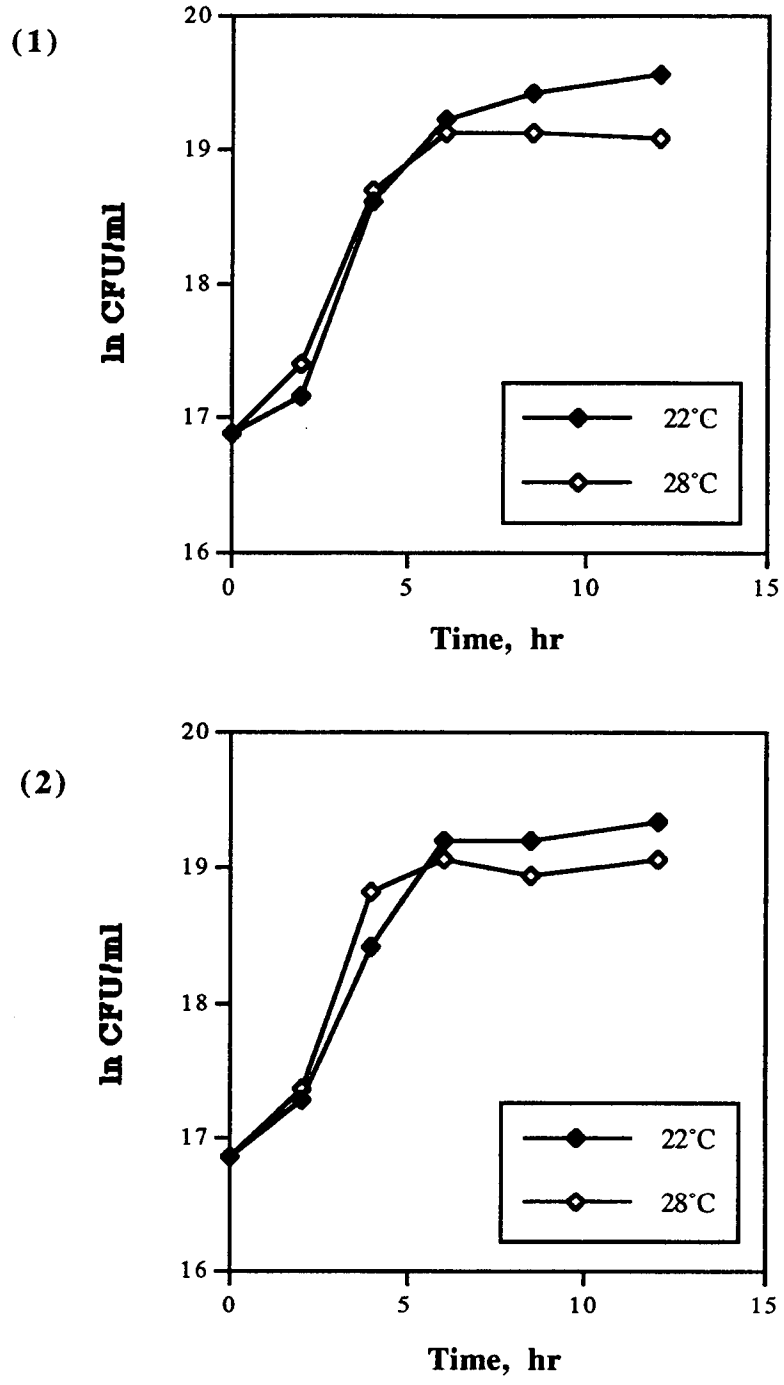

Figure 4.4 Growth of *Leu. cremoris* 91404 in (1) milk, and (2) milk supplemented with 0.2% citrate.

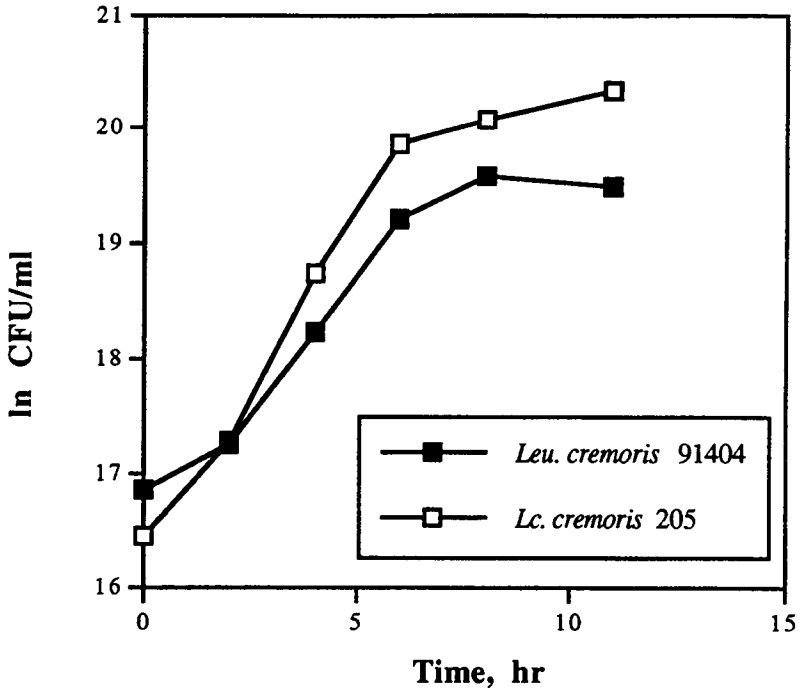

Figure 4.5 Growth of *Leu. cremoris* 91404 and *Lc.cremoris* 205 in mixed culture (11%RSM, 22°C).

Experimental buttermilks

The process used to prepare experimental buttermilk was based on commercial practice. Traditionally, manufacture of cultured buttermilk includes inoculation of pasteurized, cooled milk with a lactic starter culture containing acid and aroma producers, incubation, known as the ripening or fermentation period, breaking the coagulum and cooling followed by bottling and distribution of the final product. Milk used for fermentation is usually deficient in citrate and needs to be fortified with sodium citrate prior to pasteurization. The Code of Federal Regulations (1990) allows the addition of 0.15 % citrate. Lowfat (1%) milk used in our experiments was fortified with 0.1% sodium citrate because of its low citrate content (0.05%). The obvious reason for milk fortification with citrate is to provide more substrate for flavor production. The inducible nature of some

enzymes for citrate metabolism in leuconostocs (Mellerick and Cogan, 1981) has been another reason for the addition of citrate prior to fermentation.

Growth of the starter culture containing *Leu. cremoris* 91404 as the aroma producer during the ripening period is presented in Figure 4.6. It can be seen that citrate utilization began as soon as growth was initiated and that the flavor substrate was completely depleted at the point when growth slowed down, i.e. after ten hours of incubation.

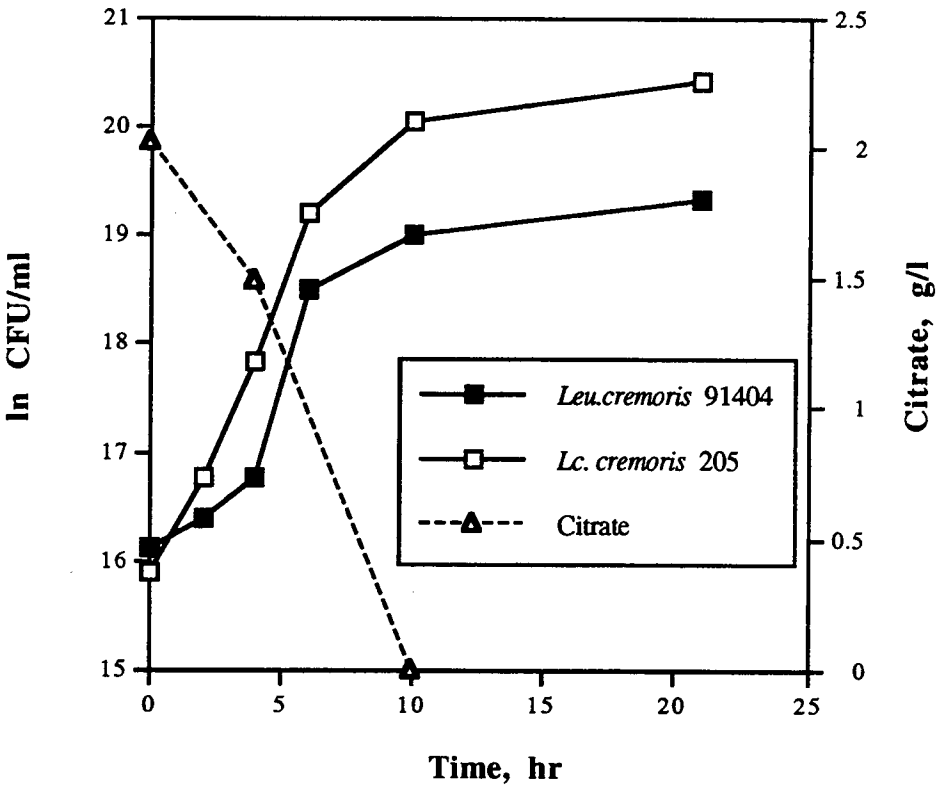

Figure 4.6 Growth of starter culture, containing *Leu. cremoris* 91404 and *Lc. cremoris* 205, during milk fermentation (buttermilk ripening).

After pH 4.6 was reached the buttermilk was fortified with additional citrate and refrigerated. This additional step represents a modification of traditional buttermilk making procedures. The results presented in Fig. 4.7 show that cultures were not actively growing during this storage period because of the low pH, low temperature and, probably,

insufficient sugar content. However, citrate utilization was evident although much slower than in ripening period.

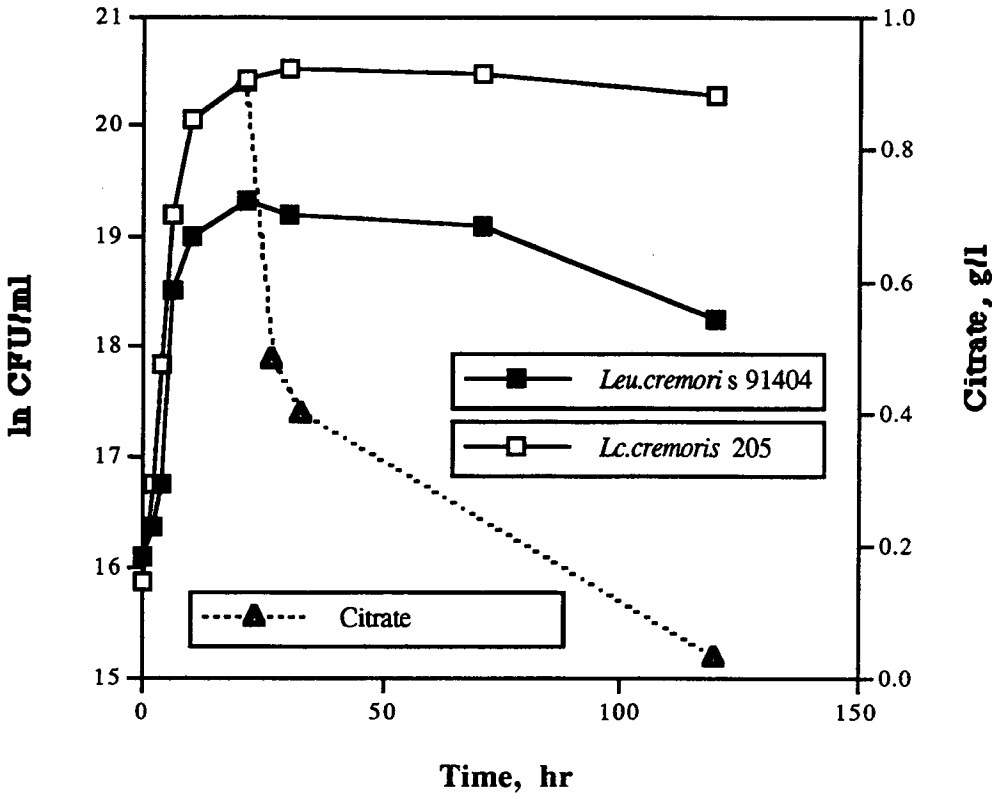

Figure 4.7 Growth of starter culture, containing Leu. *cremoris* 91404 and *Lc. cremoris* 205, during ripening and storage of experimental buttermilk.

Citrate was more readily utilized by mixed cultures than by pure cultures of *Leu. cremoris* cultivated under the same experimental conditions (Fig. 4.8). In mixed cultures, citrate utilization was facilitated by acidic conditions (pH 4.6) created by the growth of *Lc. cremoris* 205. After five days of refrigeration, the citrate content in buttermilk prepared with mixed cultures was 0.034% compared with 0.38% in milk fermented by pure *Leu. cremoris* cultures.

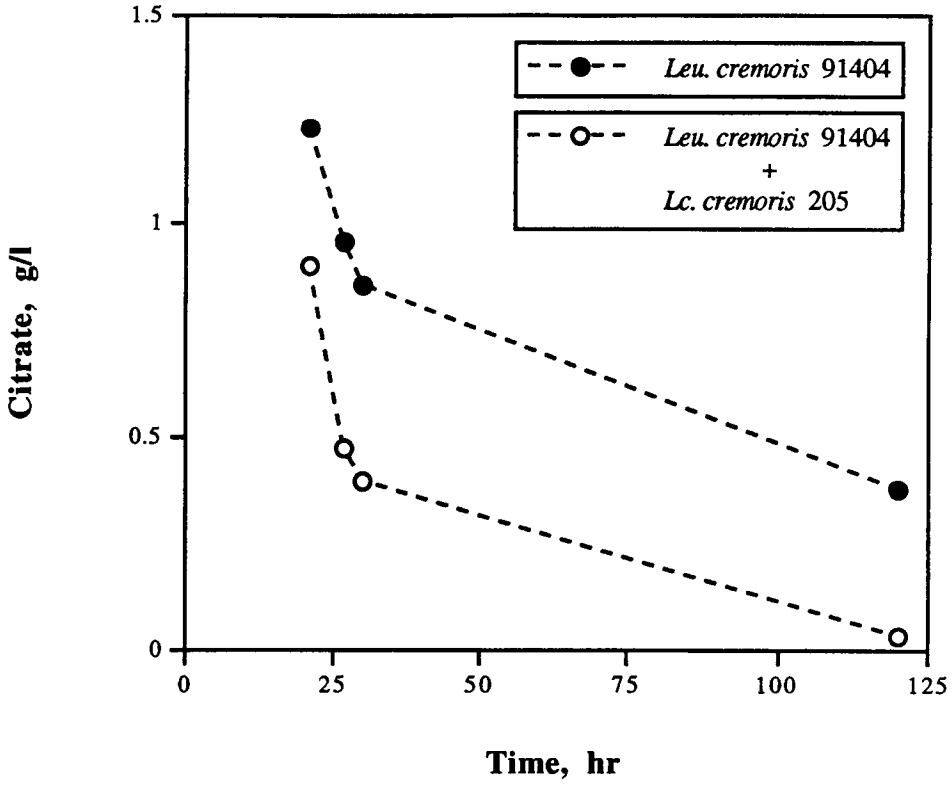

Figure 4.8 Citrate utilization by *Leu. cremoris* 91404 in pure, and in mixed culture with *Lc. cremoris* 205 during refrigeration. Cultures were preincubated in lowfat milk supplemented with 0.1% citrate at 22°C for 21 hr, then fortified with citrate and stored at 5°C for 5 days.

Citrate, added after growth ceased and favorable acidic conditions developed, was expected to act as precursor of diacetyl and acetoin, to stimulate α-acetolactate synthase by internal accumulation of pyruvate, and to stabilize the level of diacetyl in cultured buttermilk by retarding eventual diacetyl reductase activity. It has been reported that citrate present after fermentation has a slightly repressive effect on diacetyl reductase associated with starter culture organisms (Hugenholtz,1993).

Slow, gentle agitation was applied during breaking the coagulum and adding citrate solution in order to provide uniform citrate distribution and incorporation of some air into the buttermilk. It has been reported by numerous investigators (Collins,1972; Kaneko et al.,1990; Bassit et al.,1993; Hugenholtz,1993; Monnet et al.,1994) that oxygen is effective in

stimulating diacetyl formation. This can be explained by stimulation of NADH oxidase that plays a part in recycling NAD, thus facilitating accumulation of diacetyl (Collins, 1972). or by providing high redox potential important for citrate conversion to diacetyl (Monnet et al.,1994), ensued by the active role of oxygen in oxidative decarboxilation of α-acetolactate, which is the mechanism postulated for diacetyl biosynthesis in leuconostocs (Seitz et al.,1963b; Hugenholtz,1993).

Internal generation of oxygen in milk treated with hydrogen peroxide and catalase was also found to enhance and stabilize diacetyl production by mixed-strain cultures known to contain *Lc. cremoris*, *Lc. diacetylactis*, and *Leu. cremoris* (Pack et al,1968a). It was interesting for us to explore the effect of *in situ* oxygen generation on diacetyl/acetoin production in milk cultures containing *Leu. cremoris* 91404 as the only aroma producer. As shown in Fig. 4.9, growth of *Leu. cremoris* 91404 in milk fortified with sodium citrate, 0.2% was not significantly affected by the hydrogen peroxide/catalase treatment of milk (k,0.425hr^{-1}; g,98 min). Effect on diacetyl production is discussed later on in this chapter. In addition to aeration, prompt cooling of the milk cultures to temperatures below 7°C, is also reported to increase diacetyl content (Pack et al.,1968b) and was regularly applied.

Concentrations of diacetyl and other volatile compounds were determined periodically by GLC during experimental buttermilk making and storage, and compared with the volatile compound content of buttermilk made with the same cultures in the traditional way.

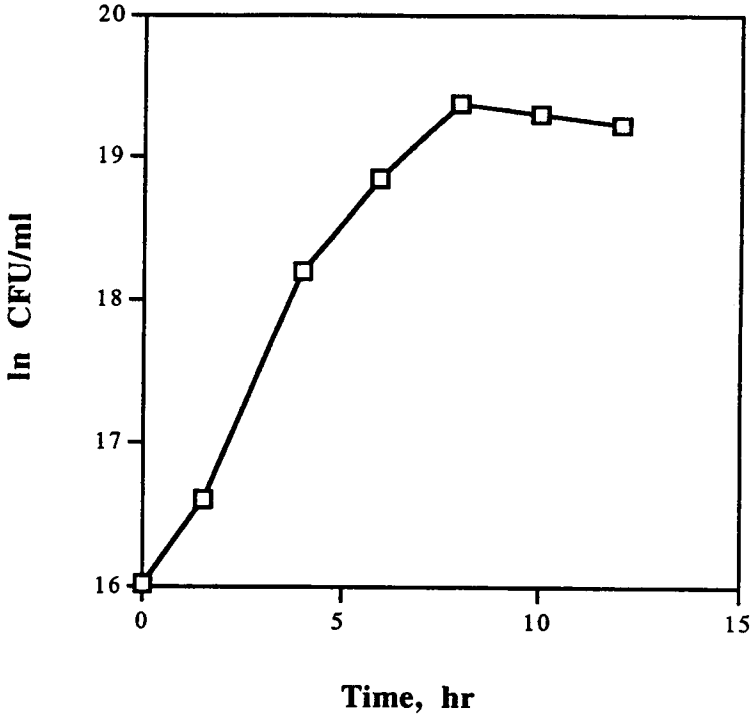

Figure 4.9 Growth of *Leu. cremoris* 91404 at 22°C, in milk supplemented with citrate and treated with hydrogen peroxide and catalase.

Gas-liquid chromatographic analysis of volatile compounds

For GLC analyses, polyaromatic material was used as the column packing matrix and it provided symmetric, sharp peaks of the tested volatiles (Fig. 4.10). Sec-butanol was used as an internal standard as it is not a known product of lactic acid bacteria and its retention time (RT) did not overlap with the other compounds of interest. Use of temperature programming also improved resolution of peaks. A removable glass inlet provided protection of the column by trapping accompanying nonvolatile substances of the sample. To increase the lifetime of the column this removable tube was changed after about 25-30 injections.

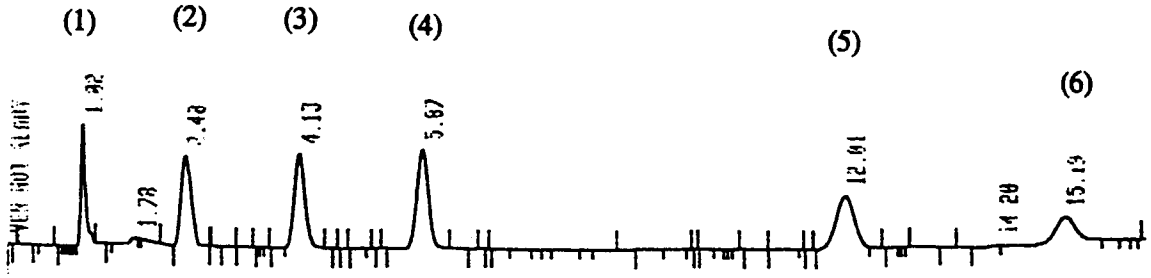

Figure 4.10 A typical chromatogram of standard solution with sec-butanol as internal standard. GC peaks: (1) acetaldehyde, RT=1.02; (2) ethanol, RT=2.48; (3) diacetyl, RT=4.13; (4) sec-butanol, RT=5.87; (5) acetoin, RT=12.01; (6) acetic acid, RT=15.19. RT=retention time, min.

Concentrations of volatile compounds present in milk cultures were extrapolated from standard curves (Fig.4.11). The standard curves were linear over the entire range of the concentrations tested. Detector response, apparent as peak areas, of ethanol and acetoin were similar. The standard curves for diacetyl and acetoin, that were of the most interest, are shown separately.

GLC peaks of volatile compounds in milk-based solutions were compared with peaks of aqueous solutions. The reproducibility of the peaks was checked to see if components in the media interfered with the analysis. Area ratios [A(aqueous)/A(milk)] for standard compounds at selected concentrations are listed in Table 4.2 along with corresponding standard deviations. Presented results are the mean values of three replications per sample.

Direct and fast GLC determination of volatile compounds in milk products without prior processing, excellent separation of tested compounds, and highly comparable response of given compounds in aqueous and milk-based solutions are advantages of the method and provide confidence that the analytical data generated are accurate.

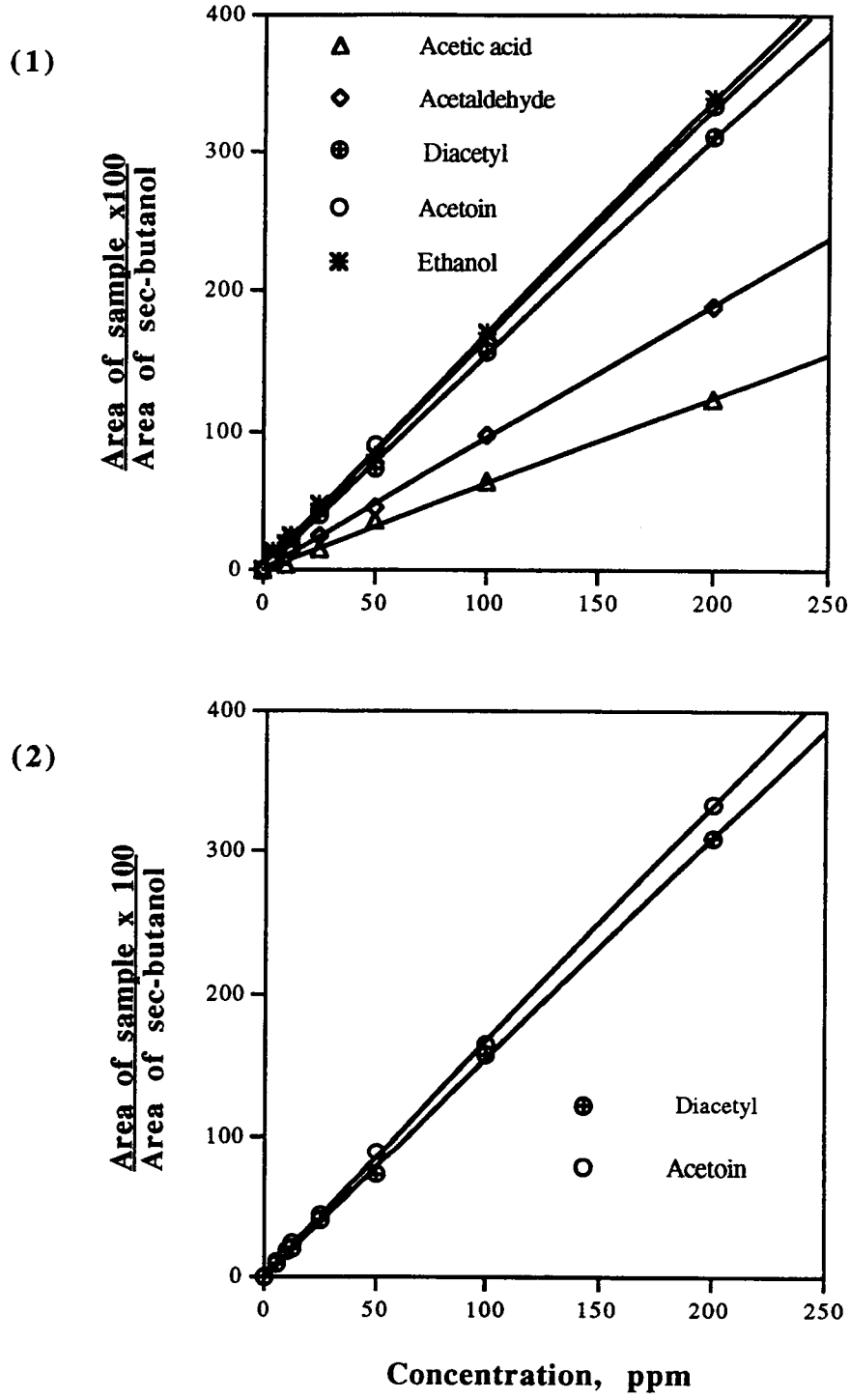

Figure 4.11 Standard curves for (1) acetic acid, acetaldehyde, diacetyl, acetoin, ethanol, and (2) diacetyl and acetoin.

Table 4.2 Peak area ratios [A(aqueous)/A(milk)] for acetaldehyde, diacetyl and acetoin.

Compound	Concentration, ppm									
	2	SD	5	SD	10	SD	25	SD	50	SD
	A(aqueous)/A(milk)									
Acetaldehyde	1.31	0.2	1.19	0.18	1.04	0.04	1.07	0.13	0.96	0.01
Diacetyl	0.97	0.05	1.05	0.17	0.96	0.07	0.95	0.04	1.08	0.08
Acetoin	NC	-	0.92	0.08	1.16	0.11	1.03	0.11	0.95	0.08

SD = standard deviation; NC = not checked

It is important to add, however, that acetic acid present in large quantity in milk fermented with *Leu. cremoris* tended to absorb on the column causing irregularly shaped peaks. Absorbed portions of acetic acid eluted during subsequent sample injections and therefore interfered with accurate quantification of acetic acid in the fermented milk. Also, in analyses of milk cultures, the packing material tended to be unstable and sometimes caused ghost peaks and baseline disturbances after serial injections of samples. Flush-runs with deionized water (0.5µl) were done to ensure that the column had no remaining material to interfere with further sample runs. Occasional conditioning at 150°C was necessary to stabilize the packing material and reduce baseline disturbances. Finally, possible inaccuracies in the determination of diacetyl by GLC analysis can be associated with instability of α-acetolactate which is presumably an intermediate in diacetyl formation. Spontaneous decarboxilation of ALA at high temperature during the analysis of diacetyl may lead to overestimation of the true diacetyl content (Jordan and Cogan, 1988).

Gas-liquid chromatograms of *Leu. cremoris* 91404 milk cultures

Leu. cremoris 91404 grown in milk produced mainly ethanol and acetic acid (Fig.4.12). High levels of ethanol are an indication of heterofementative metabolism (Thornhill and Cogan,1984), while acetate production is typical for citrate utilization (Drinan et al.,1976). As previously shown *Leu. cremoris* utilized citrate under both neutral and acid conditions, but diacetyl and acetoin were produced only under acidic conditions in the presence of surplus citrate. Figure 4.13 represents the chromatogram of *Leu. cremoris* 91404 grown under the conditions specified by Mather and Babel (1959). Production of very high concentrations of diacetyl and acetoin, 75 and 326 ppm respectively, could be attributed to citric acid fortification during the stationary growth phase, and to the extreme acidic conditions that are favorable for citrate consumption initiated by action of citrate permease active at pH below 6.0.

The influence of internal oxygen generation on diacetyl/acetoin production by *Leu. cremoris* was investigated in milk treated with hydrogen peroxide and catalase prior to inoculation. This treatment was experimentally found to enhance and stabilize diacetyl in mixed strain cultures containing *Lc. diacetylactis* and *Leu. cremoris* as aroma producers (Pack et al.,1968). Recently, Monnet et al. (1994) showed that diacetyl production by *Lc. diacetylactis* was due to the chemical oxidative decarboxylation of ALA and is favored by aerobic conditions. According to these authors, diacetyl is produced only at high redox potential as the result of this chemical mechanism. In our experiments, fermentation of hydrogen peroxide-catalase treated milk by *Leu. cremoris* 91404 resulted in diacetyl and acetoin production after prolonged incubation. Single-strain culture produced 10 ppm of diacetyl and 9 ppm of acetoin after 48 hr of incubation at 22°C, and 8 ppm of diacetyl and 22 ppm of acetoin after 48 hr of incubation at 30°C. Interestingly, pH of the culture in treated milk after 48 hr incubation was lower (pH 5.4) than the pH of the control culture in untreated milk (pH 5.9), indicating better growth of strain 91404 and/or more acid production in the presence of oxygen. Furthermore, pH 5.4 is close to pH optimum for

citrate metabolism (Gilliland,1984). These results indicate the importance of oxygen level and redox potential for diacetyl and acetoin production in *Leu. cremoris*.

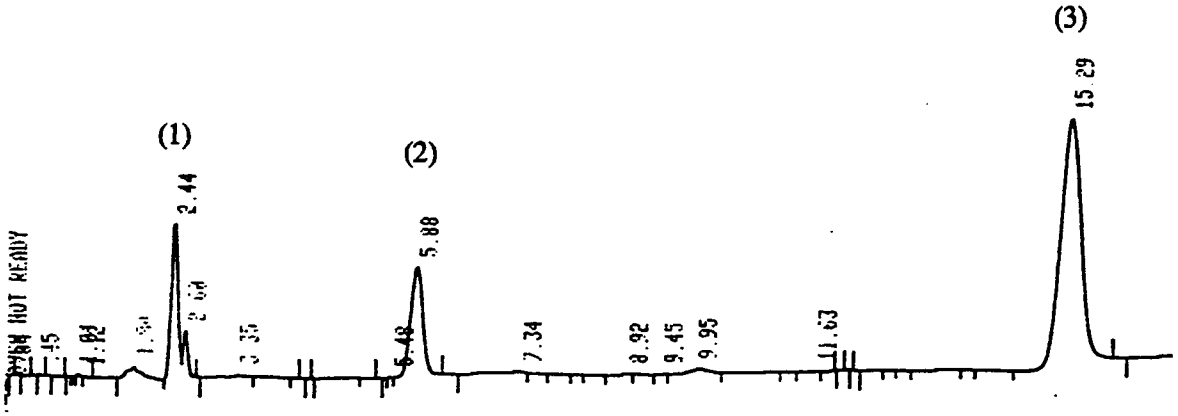

Figure 4.12 Chromatogram of pure milk culture of *Leu. cremoris* 91404. GC peaks were identified as: (1) ethanol, RT=2.44; (2) sec-butanol (IS), RT=5.88; (3) acetic acid, RT=15.29. RT=retention time, min.

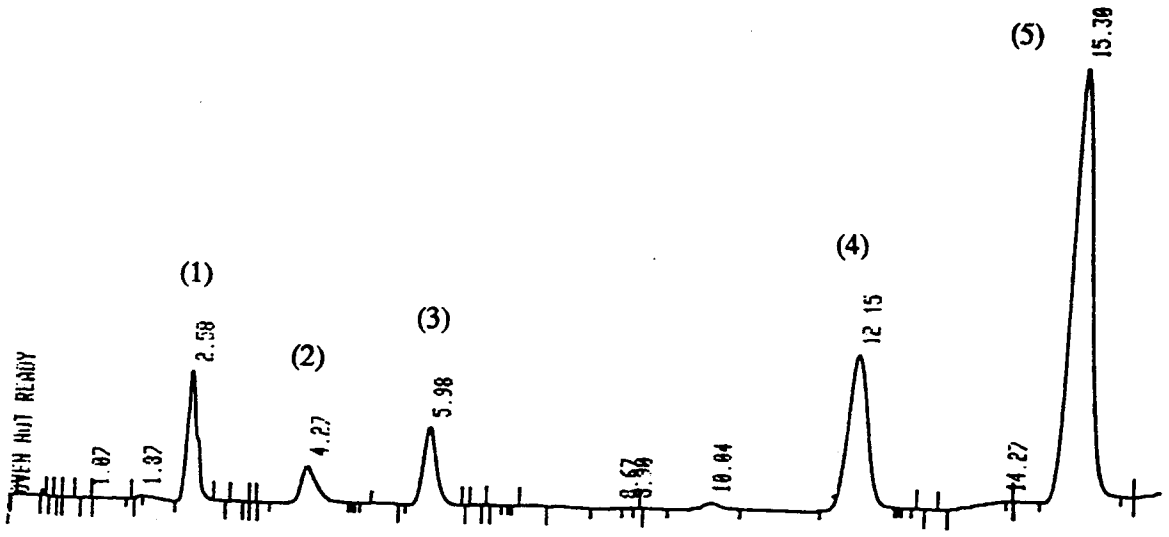

Figure 4.13 Chromatogram of milk culture of *Leu. cremoris* 91404 preincubated for 24 hr and subsequently acidified with citric acid to pH 4.3. GC peaks were identified as: (1) ethanol, RT=2.58; (2) diacetyl, RT=4.27; (3) sec-butanol (IS), RT=5.98; (4) acetoin, RT=12.15; (5) acetic acid, RT=15.30. RT=retention time, min.

Gas-liquid chromatograms of mixed cultures

Lc. cremoris strains, 205 and 382, used to acidify milk in mixed strain studies were unable to utilize citrate, which was verified on citrate-indicator agar and by enzymatic analyses of milk cultures. Commonly, no detectable amounts of diacetyl and variable amounts of acetoin (0 - 40 ppm) were produced during fermentation by *Leu. cremoris* 91404 under acidic conditions created by growth of *Lc. cremoris* in the mixed cultures (Fig. 4.14). The lack of diacetyl production may be explained by directing the citrate metabolism to products different from dicarbonyl and/or, as was found by Monnet et al. (1994), by rapid drop of redox potential at the beginning of the mixed strain fermentation resulting in absence of oxidative decarboxilation of ALA to diacetyl.

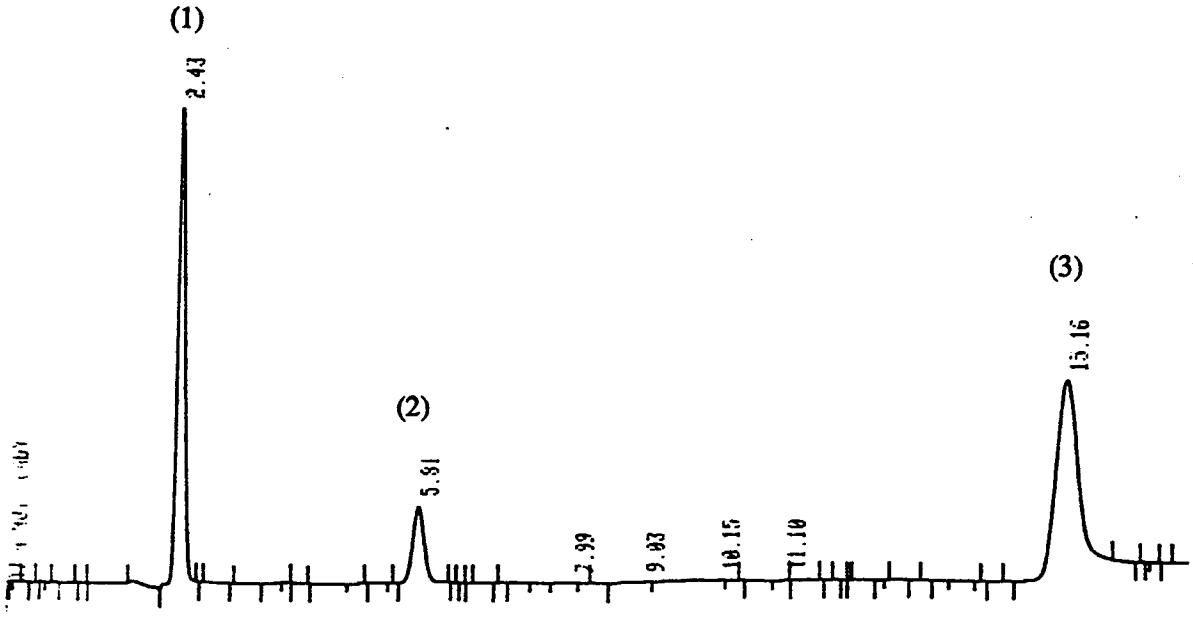

Figure 4.14 Chromatogram of milk culture of *Leu. cremoris* 91404 acidified with *Lc. cremoris*. GC peaks were identified as: (1) ethanol, RT=2.43; (2) sec-butanol (IS), RT=5.81; (3) acetic acid, RT=15.16. RT=retention time, min.

Effect of oxygen level on diacetyl production in mixed cultures was investigated after hydrogen peroxide-catalase treatment of milk, and in agitated milk cultures. After hydrogen-peroxide treatment of milk, maximal diacetyl (avg. 5ppm) and acetoin (avg. 30.5 ppm) concentrations were reached after 10 hr of incubation (Fig. 4.15). Buildup of diacetyl and acetoin by the 10th hour of fermentation paralleled the depletion of citrate and pH decline to 5.3. On the contrary, in the control experiment (no milk treatment) no diacetyl or acetoin was detected after 10 hr of incubation. Production of diacetyl and acetoin in hydrogen peroxide-catalase treated milk in this early stage could be attributed to elevation of the redox potential by internal generation of oxygen. This presumption is in agreement with the explanation of Monnet et al. (1994) that low or no diacetyl production in mixed cultures is the result of a rapid drop in redox potential at the beginning of growth leading to complete citrate consumption without the oxidative decarboxylation of ALA. However, at the end of ripening period no diacetyl was detected and acetoin was present in lower concentrations (avg. 16.1 ppm) indicating the destruction of these compounds.

In agitated milk cultures, increases in diacetyl and acetoin production continued up to the end of fermentation. After 12 hr of fermentation, diacetyl and acetoin concentrations in agitated cultures were 1.8 ppm and 57 ppm respectively, and after 24 hr they were 3 and 70 ppm (Fig.4.16). Another means to incorporate air during fermentation is by flushing air through the culture. According to the results of Hugenholtz and Starrenburg (1992) simple aeration stimulated α-acetolactate production in starter cultures used for dairy fermentations requiring the production of the flavor compound, diacetyl.

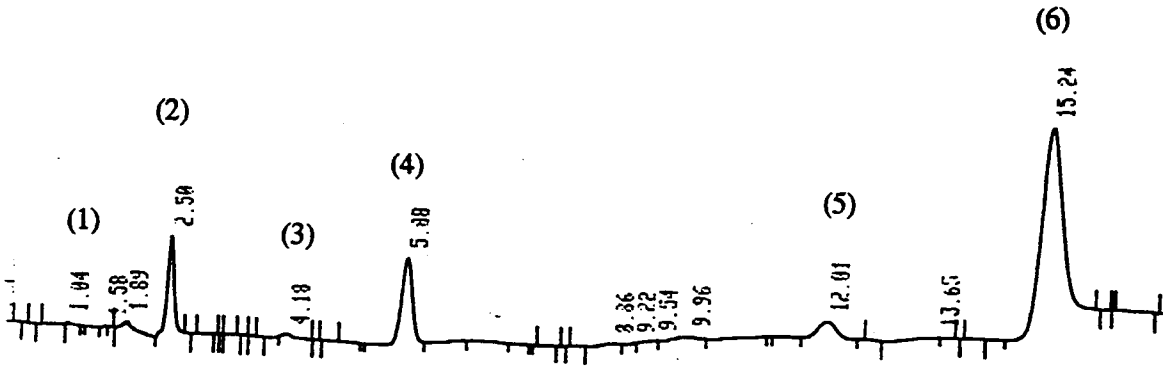

Figure 4.15 Chromatogram of mixed culture containing *Leu. cremoris* 91404 and *Lc. cremoris* 205 after 10 hr fermentation in milk treated with hydrogen-peroxide and catalase. GC peaks were identified as (1) acetaldehyde, RT=1.04; (2) ethanol, RT=2.50; (3) diacetyl, RT=4.18; (4) sec-butanol (IS), RT=5.88; (5) acetoin, RT=12.01; (6) acetic acid, RT=15.24. RT=retention time, min.

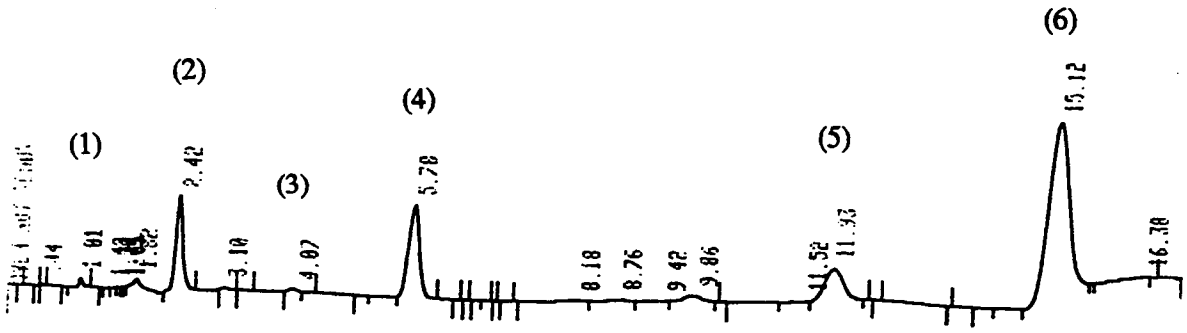

Figure 4.16 Chromatogram of continuously agitated mixed culture, containing *Leu. cremoris* 91404 and *Lc. cremoris* 205, after 24 hr incubation on the shaker. GC peaks were identified as: (1) acetaldehyde, RT=1.01; (2) ethanol, RT=2.42; (3) diacetyl, RT=4.07; (4) sec-butanol (IS), RT=5.78; (5) acetoin RT=11.93; (6) acetic acid, RT=15.12. RT=retention time, min.

Although we haven't studied in detail the effect of hydrogen peroxide-catalase milk treatment and the effect of agitation on citrate metabolism, it is obvious from the preliminary results presented that oxygen level and redox potential play important roles in diacetyl production by leuconostocs in association with lactococci. Additionally, kinetic studies on diacetyl production related to redox potential, are necessary. Nevertheless, we have chosen to study more thoroughly the effect of citrate level on diacetyl/acetoin production in mixed culture because of its likely practical use in actual cultured dairy product manufacture.

Gas liquid chromatographic analysis of experimental buttermilk

Fortification of buttermilk with sodium citrate after ripening resulted in enhanced diacetyl and acetoin production. Rapid citrate uptake and conversion to diacetyl and acetoin took place in all buttermilks that were supplemented with citrate after fermentation with *Leu. cremoris* 91404 and *Lc. cremoris* strains was completed (Fig. 4.17-4.19). Excess of citrate, low pH, sufficient number of active, non-growing leuconostocs, incorporation of air by shaking during curd breaking and 2nd citrate addition, and cooling the buttermilks to 5°C had favorable effects on flavor development in experimental buttermilk during storage. For example, refrigerated buttermilk made with *Leu. cremoris* 91404 and *Lc. cremoris* 205 and 352 contained (ppm): acetaldehyde, 1.7, 2.2; diacetyl, 5.7, 9.4; acetoin, 225.2, 220.4; ethanol, 158.4, 146.2; and acetic acid, approximately 1083, 1041; and a diacetyl/acetaldehyde ratio, of 3.3, 4.3; after one and two weeks of storage, respectively.

Citrate uptake was stimulated by low pH because of citrate permease pH dependency and since a considerable fraction of the citrate (pK_a = 3.14, 4.77 and 5.40) in buttermilk at pH 4.5-4.6 is present in the uncharged, acidic form, which can easily diffuse through the bacterial membrane (Hugenholtz,1993). In addition to providing the precursor of diacetyl under appropriate conditions, citrate was, most likely, involved in induction of citrate lyase and α-acetolactate synthase (ALS). Hugenholtz and Starrenburg (1992) reported 20-fold increase in specific activity of citrate lyase and 2-10-fold induction of ALS

upon addition of citrate to *Leuconostoc* cultures. Fortification with precursor also provides a safety margin in preventing flavor loss by repressing diacetyl reductase. High concentrations of acetoin in buttermilk may have an inhibitory effect on enzyme activity, also (Hugenholtz, 1993). The higher affinity of diacetyl (acetoin) reductase for acetoin than for diacetyl together with non-competitive inhibition of enzyme activity by acetoin is probably another reason for the low rates of diacetyl reduction in buttermilk. Cooling cultured products to refrigeration temperature also arrests the destruction of diacetyl by retarding diacetyl reductase activity. Although diacetyl reduction was not expected to present the major problem in our experiments considering the low diacetyl reductase activity of the strain 91404, it is important to consider these aspects of citrate metabolism since a variety of starters are used in buttermilk production and contamination with psychrotrophs, that have high DR activity, is not uncommon in commercial practice (Wang and Frank,1981).

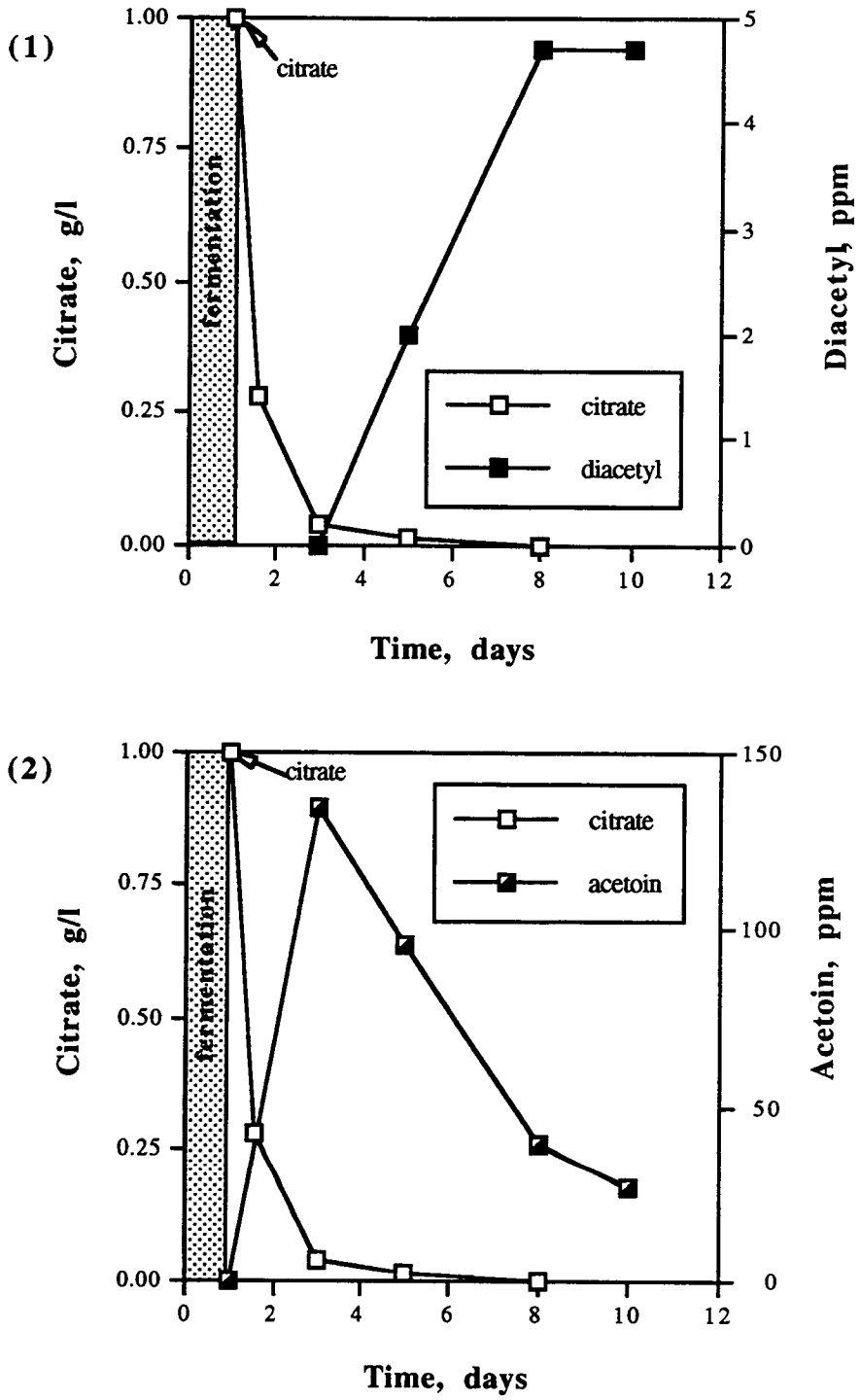

Figure 4.17 Citrate utilization and production of (1) diacetyl and (2) acetoin during storage of experimental buttermilk after fortification with sodium citrate. Starter culture: *Leu. cremoris* 91404 and *Lc. cremoris* 205.

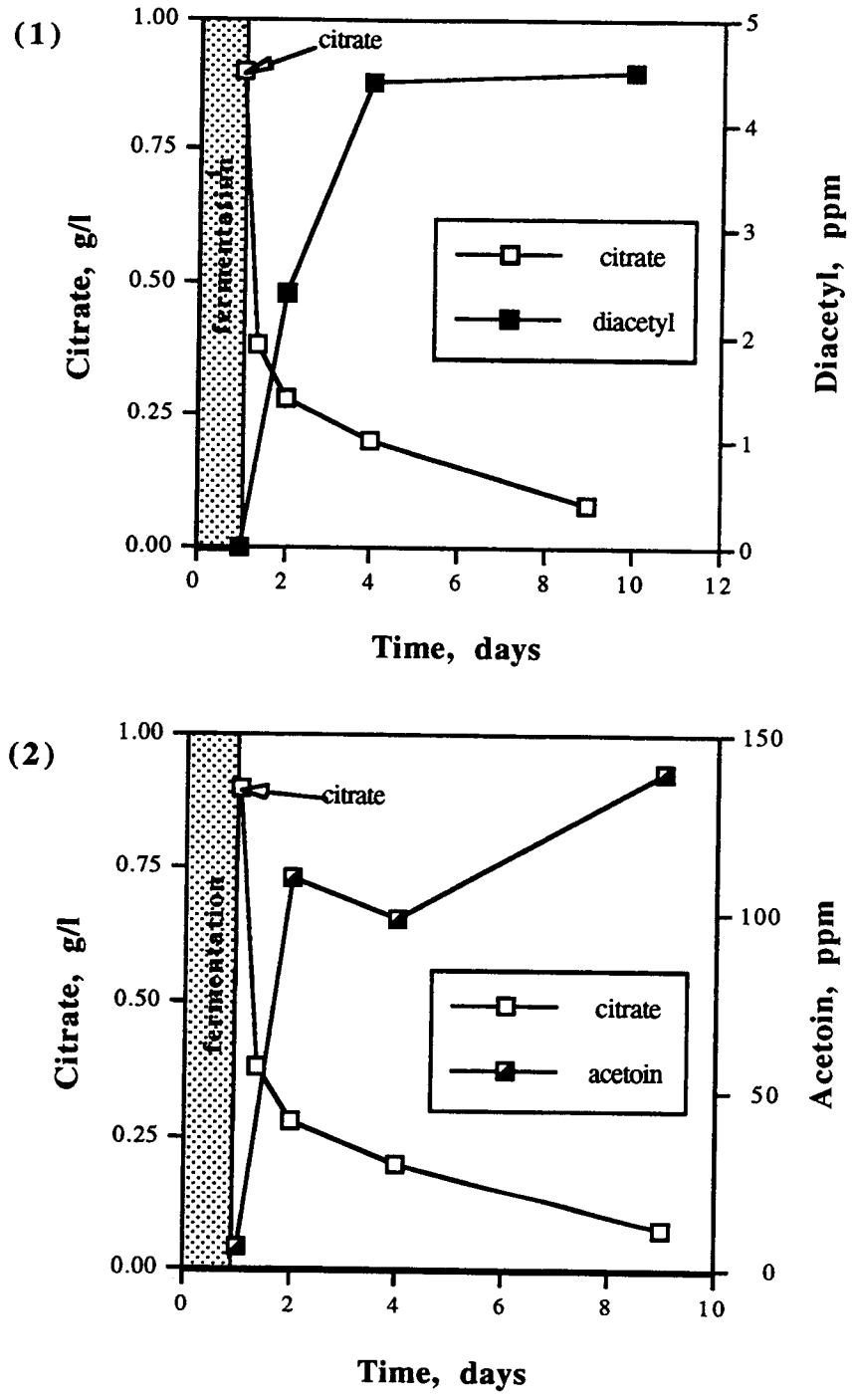

Figure 4.18 Citrate utilization and production of (1) diacetyl and (2) acetoin during storage of experimental buttermilk after fortification with sodium citrate. Starter culture: *Leu. cremoris* 91404 and *Lc. cremoris* 352.

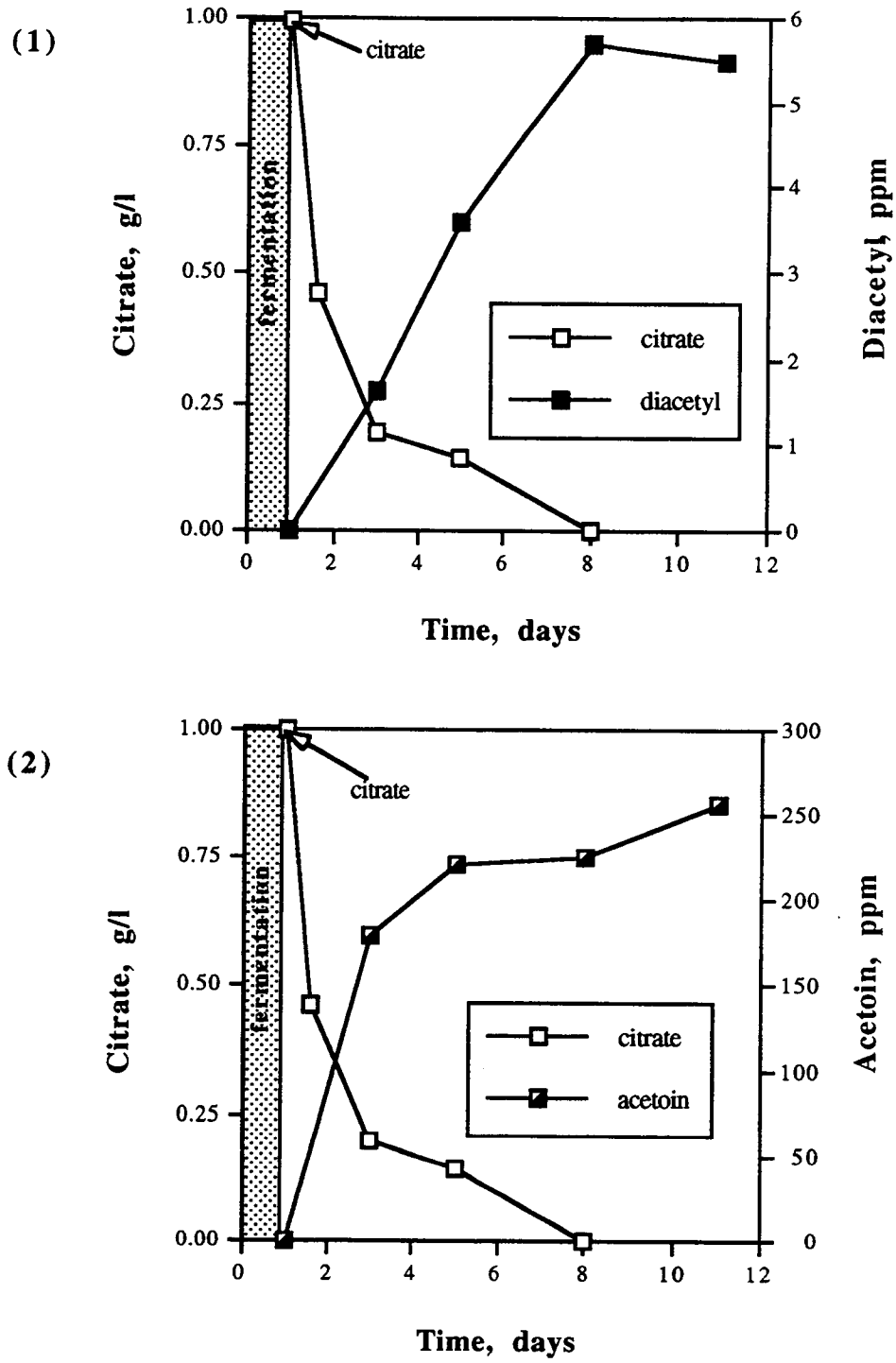

Figure 4.19 Citrate utilization and production of (1) diacetyl and (2) acetoin during storage of experimental buttermilk after fortification with sodium citrate. Starter: *Leu. cremoris* 91404, and *Lc. cremoris* 205 and 352.

Additional fortification with citric acid showed the same results as added sodium citrate (Fig.4.20 - 4.22). However, the addition of citric acid was limited by its effect on pH of buttermilk and from that point of view was not applicable for commercial production of flavorful buttermilk.

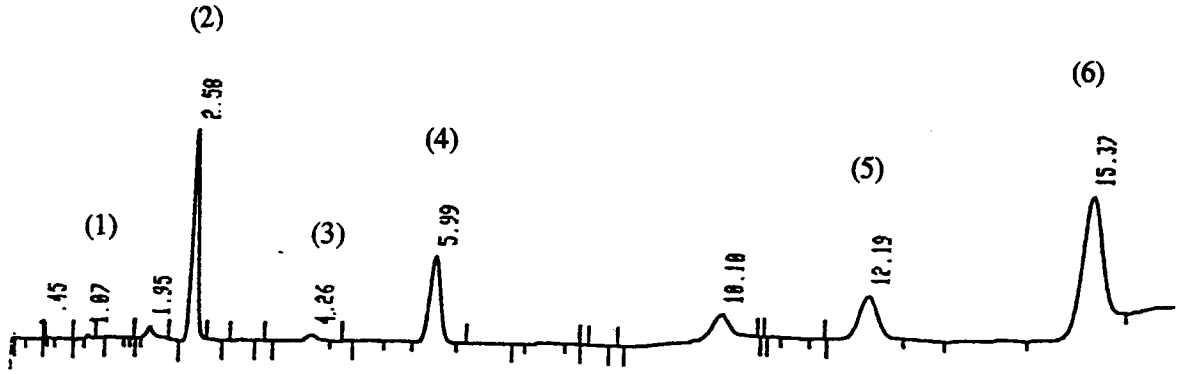

Figure 4.20 Chromatogram of experimental buttermilk made with *Leu. cremoris* 91404 and *Lc. cremoris* 205 and 352, stored at 5°C for three days after delayed fortification with citric acid. GC peaks were identified as: (1) acetaldehyde, RT=1.07; (2) ethanol, RT=2.58; (3) diacetyl, RT=4.26; (4) sec-butanol (IS), RT=5.99; (5) acetoin, RT=12.19; (6) acetic acid, RT=15.37. RT=retention time, min.

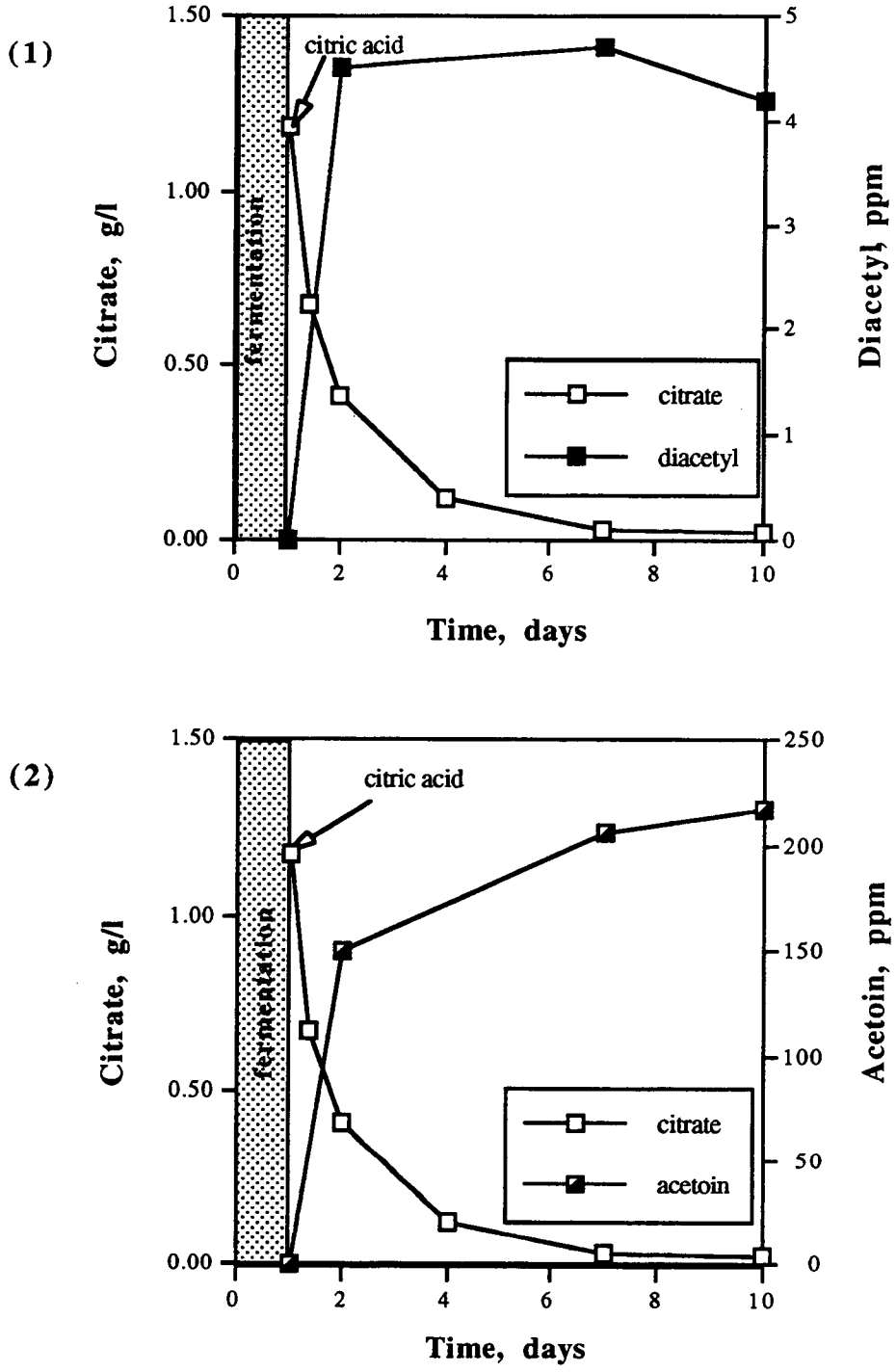

Figure 4.21 Citrate utilization and production of (1) diacetyl and (2) acetoin during storage of experimental buttermilk after fortification with citric acid. Starter culture: *Leu. cremoris* 91404 and *Lc. cremoris* 205.

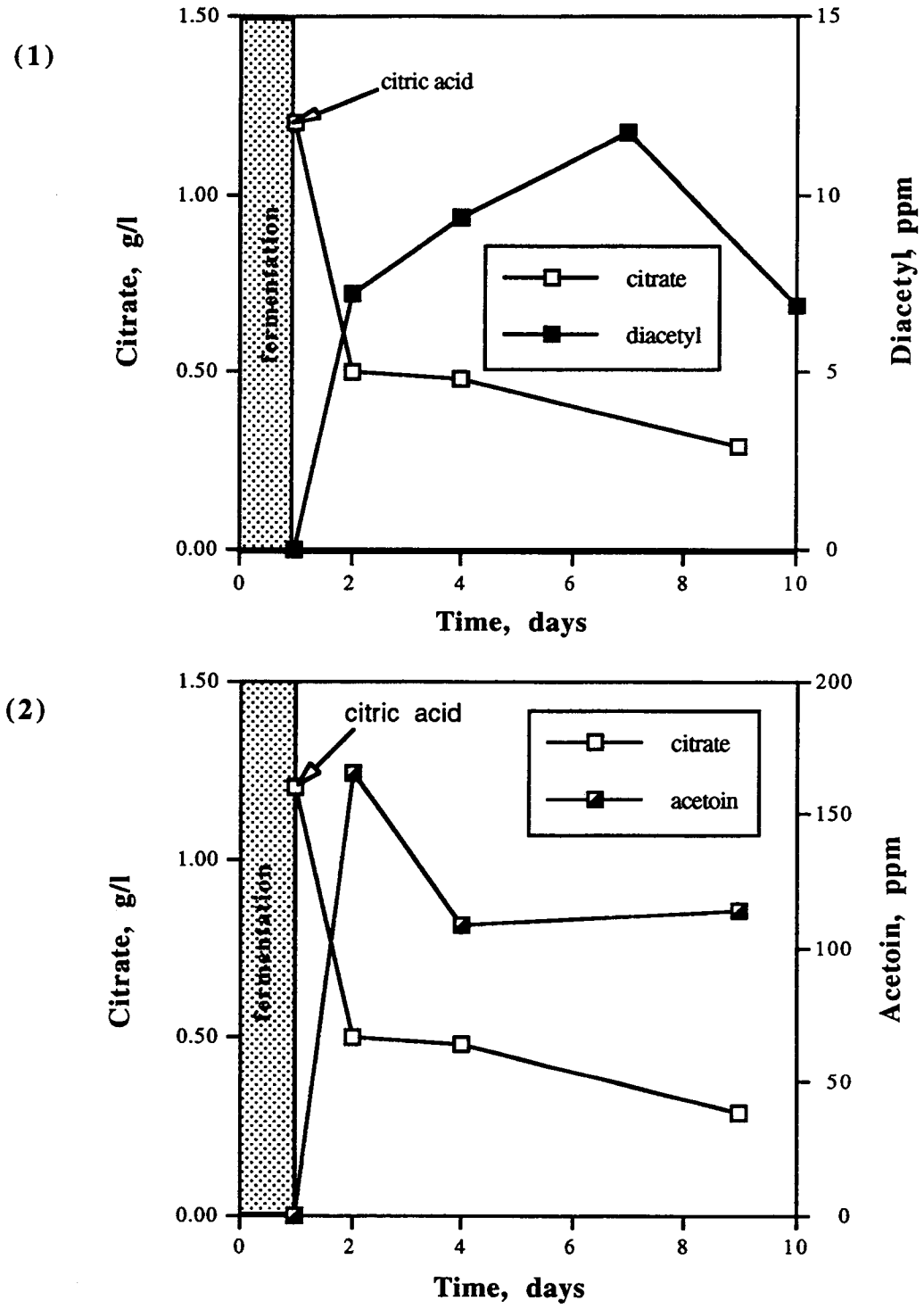

Figure 4.22 Citrate utilization and (1) diacetyl and (2) acetoin production during storage of experimental buttermilk after fortification with citric acid. Starter culture: *Leu. cremoris* 91404 and *Lc. cremoris* 352.

4.4 Conclusions

1. *Leu. cremoris* 91404 metabolized citrate under neutral and acidic conditions. Citrate utilization under initial neutral conditions corresponded to the active growth of starter culture.

2. Gas-liquid chromatographic analysis was found suitable for direct determination of volatile compounds in buttermilk produced by *Leu. cremoris* .

3. No diacetyl was detected in buttermilk made in the traditional commercial way with mixed cultures containing *Leu. cremoris* 91404 and *Lc. cremoris* 205.

4. Diacetyl was produced only under acidic conditions in the presence of citrate surplus. Fortification of ripened buttermilk with sodium citrate resulted in significant increase in diacetyl and acetoin production during buttermilk storage at 5°C.

5. Beside the role of added citrate in diacetyl production as precursor and as inducer of enzymes involved in citrate metabolism, appropriate conditions such as: low pH, sufficient number of active non-growing aroma producers, air incorporation during curd breaking, and low temperature of storage, facilitated channeling the citrate metabolism toward flavor production and its conservation during two weeks of storage.

6. Preliminary results showed that internal generation of oxygen attained by hydrogen peroxide-catalase treatment of milk, and aeration of buttermilk by continuous shaking the buttermilk during fermentation period stimulated the diacetyl and acetoin production by *Leu. cremoris* 91404.

CHAPTER 5

Evaluation and comparison the quality of commercial and experimental buttermilks

5.1 Introduction

Cultured buttermilk is very attractive dairy product due to attributes such as: high nutrition value, low fat content, low(er) lactose content, sodium free product unless salted, readily and easily digested milk product (Vedamuthu,1977). Desired cultured buttermilk is a clean, mildly acid, smooth, slightly aromatic and carbon dioxide containing, refreshing liquid milk drink.

Although technologically, buttermilk is the simplest of the cultured dairy products to produce, sampling of brands available today indicate that many of them do not meet the acceptable industry standards for body, texture, flavor and freshness (Keenan et al.,1968; Vasavada and White, 1979; Vedamuthu,1985). According to Frank (1984) most common defects of cultured buttermilks are "lack of fine flavor" (flat flavor), "high acid" (sharp flavor), and "unclean" (off flavors). Use of *Lc. diacetylactis*, as aroma producer in starter mixture, was related to excessive amounts of acetaldehyde, which imparts a "green" and harsh flavors (Vedamuthu, 1977).

During recent years there has been more demand for information pertaining to the manufacture of a uniform, good quality buttermilk. The production of high quality cultured buttermilk requires the use of fresh milk, the selection of good cultures used at the proper inoculation level, culturing at 21-23°C until the proper amount of flavor is produced, and using milk with an adequate citrate content. Maintaining good buttermilk flavor throughout shelf life of the product involves keeping diacetyl reductase activity to a minimum. In addition to the delicate flavor, high quality buttermilk must have a specific texture and body. The importance of the physical characteristics of fermented milks is reflected by the increasing use of polysaccharide-producing organisms which are incorporated into multiple

strain starters used for the production of many different dairy products (Wacher-Rodarte et al.,1993). Texture also plays an important role in flavor perception, because increased viscosity will change the mouthfeel of the product allowing flavor volatiles to remain in the mouth for a longer period.

The goal of this study was to generate further information about the quality of commercial buttermilks. Organoleptic, chemical and microbial quality of the samples were compared with an experimental buttermilk. Polysaccharide-producing starters were used to improve the texture and appearance of experimental buttermilk. The protocol for manufacturing flavorful buttermilk is recommended in the conclusion.

5.2 Material and methods

Samples

Commercial buttermilk samples from six different regional dairies were purchased from retail outlets in Corvallis, OR. These samples were coded with two numbers. First number stands for the brand as follows: 1=Lochmead, 2=Fred Meyer, 3=Albertsons, 4=EchoSpring, 5=Lucerne, and 6=Darigold. Second number stands for the batch as follows: 1=september, 1993; 2=october, 1993; 3=november, 1993; 4=april, 1994; 5=june, 1994; 6=august, 1994. Portions of each sample were used for organoleptic evaluation, bacteriological and GLC analyses. All analyses were on the day of purchase and all samples were well within the expiration date for consumption.

Experimental buttermilk

Portions (600 ml) of 1% fat (Fred Meyer) or nonfat milk (Darigold) fortified as needed with 0.1% sodium citrate, were pasteurized in glass milk bottles by steaming for 45 min. Two types of mixed cultures were used for inoculation: 1) *Leu. cremoris* 91404 (3%) and *Lc. cremoris* 205 (1%); 2) *Leu. cremoris* 91404 (3%), *Lc. cremoris* 205 (0.75%) and

Lc. cremoris 352, ropy (0.75%). *Leu. cremoris* 91404 was grown in 11% RSM supplemented with 0.3% yeast extract at 28°C for 24 hr. *Lc. cremoris* strains were grown in 11% RSM at 22°C for 18 hr. Inoculated milk was incubated at 22°C until pH 4.6-4.7 was reached (about 18 hr). At the end of ripening period the buttermilk was cooled in ice water and shaken gently to break the curd. Immediately, sodium citrate was added to a final level of 0.1%, from a sterile 30% stock solution, and shaking was repeated. After cooling, citrate fortification and curd breaking, the buttermilk was stored at 5°C. Samples were taken after four days of storage and analyzed for pH, content of volatile compounds and organoleptic characteristics.

Gas-liquid chromatographic analysis

The amounts of volatile compounds in buttermilk samples were measured by direct GLC using a model 5170A, Hewlett Packard gas chromatograph equipped with a flame ionization detector and coupled with a 3390A, Hewlett Packard integrator. The glass column used was 80/120 Carbopack B AW/6.6% Carbowax 20 M (Supelco chromatography products, Inc., Bellefonte, PA), 2m long and 2mm in diameter. Nitrogen gas was used as carrier gas at a flow rate of 20 ml/min at 60 psi. During combustion, hydrogen and compressed air were set at 34 and 26 psi, respectively. The column temperature was programmed to increase from 90 to 130°C at rate of 2°C/min, while temperatures of the injection port and detector were set at 170 and 150°C, respectively.

Concentrations of volatiles were determined by the Internal Standard calculation for the integrator used. Sec-butanol was used as internal standard (IS) at a final concentration of 50 ppm. Standard solutions were prepared daily from stock solutions. Fresh stock solutions of standards (acetaldehyde, ethanol, diacetyl, acetoin and acetic acid) were prepared each week by dissolving the volatiles (Aldrich, Milwaukee, WI) in Millipore filtered water to give a 1000 ppm solutions. Fine tuning of the GC with fresh standards each day was part of the protocol for GLC analysis. Standard curves were plotted from

fresh aqueous solutions of standards and were used to quantitate the amounts of volatiles in buttermilk samples (Thornill and Cogan,1984).

Samples of commercial and experimental buttermilks were prepared as follows. Samples were cooled on ice, then clarified by centrifugation at 13,000 rpm for 10 min and filtered through 0.45 μm-pore-size filter. Filtrates were diluted 1:1 with 1 mM sec-butanol, and 0.5 μl of this mixture was injected into the gas chromatograph.

Bacteriological analysis

Buttermilk samples, diluted according to procedures described in the *Compendium of Methods for Microbiological Examination of Foods* (1976), were plated for viable cell counts on both MRS agar and MRS agar containing 30μg/ml vancomycin. All cultures plates were incubated at 28°C for 24-36 h. *Leuconostoc* counts were based on number of colonies (CFU/ml) grown on MRS agar supplemented with vancomycin, while *Lactococcus* counts were determined by subtracting *Leuconostoc* counts from total viable cell counts obtained on MRS agar.

One batch (august, 1994) of pasteurized lowfat milks from six regional dairies, coded the same as corresponding buttermilks from 1 to 6, was checked for psychrotrophic bacteria count performed by plating the dilutions of milk samples on crystal violet tetrazolium agar (CVT). CVT counts were determined after 5 days incubation at 22°C according to the procedure described in the *Compendium of Methods for Microbiological Examination of Foods* (1976).

Organoleptic evaluation

Buttermilk samples were presented to five experienced panelists. Panelist were asked to record flavor, texture, mouthfeel, acidity and preferences for particular sample.

5.3 Results and Discussion

Characteristics of commercial buttermilks

Results of GLC analyses of six buttermilk batches from six Oregon dairies are shown in Tables 5.1-5.6. Rather large variations in concentrations of volatile compounds are apparent. This is not surprising since consistency in flavor production in cultured buttermilk is still difficult to attain in commercial operations. In the samples that we analyzed the ratio of diacetyl to acetaldehyde showed considerable variation. According to Lindsay (1965) flavor balance is controlled by the ratio of these two compounds. However, there appears to be lack of correlation between this ratio and overall flavor evaluation. Further, many samples exhibited off-flavors such as cooked, unclean and green apple flavor. Green apple flavor correlated well with high acetaldehyde concentration, indicating the practice of using *Lc. diacetylactis* in starter mixtures for buttermilk. Most common defect mentioned was flat flavor, even in samples that showed significant levels of diacetyl (samples 1/3, 1/5, 3/2). Nevertheless, a higher flavor score appeared to be associated with high diacetyl and acetoin. Chromatographic analyses demonstrated the complexity of buttermilk samples and showed the presence of many different compounds. Some of them, like diacetyl, acetaldehyde, alcohol, acetic acid, carbon dioxide, as well as the other compounds which were not identified, are associated with the delicate flavor of buttermilk.

Table 5.1 Acidity (pH), gas liquid chromatographic analyses and comments on organoleptic characteristics of commercial buttermilk #1.

Sample (Brand/ batch)	pH	Acetaldeh. [ppm]	Diacetyl [ppm]	Acetoin [ppm]	Ethanol [ppm]	Diacetyl: Acetaldeh. ratio	Organoleptic comments
1/1	4.5	3.1	3.8	401.2	22.5	1.2:1	green flavor, grainy texture
1/2	4.5	2.5	n.d.	353.3	25.7	–	flat flavor smooth texture
1/3	4.6	3.5	5.2	344.6	22.8	1.5:1	flat flavor low acid
1/4	4.6	10.0	n.d.	514.4	37.2	–	green flavor smooth, thick
1/5	4.5	4.4	6.8	427.3	15.3	1.5:1	flat flavor smooth texture
1/6	4.6	2.0	0.7	424.9	12.5	0.3:1	N.C.

n.d.=not detected; N.C.=not checked

Table 5.2 Acidity (pH), gas liquid chromatographic analyses and comments on organoleptic characteristics of commercial buttermilk #2.

Sample (Brand/ batch)	pH	Acetaldeh. [ppm]	Diacetyl [ppm]	Acetoin [ppm]	Ethanol [ppm]	Diacetyl: Acetaldeh. ratio	Organoleptic comments
2/1	4.3	2.5	4.6	249.9	1427.7	1.8:1	nice flavor smooth texture higher acid
2/2	4.4	2.5	3.4	62.1	26.05	1.4:1	scorched flavor slightly salty weak body
2/3	4.4	3.9	2.6	20.2	28.1	0.7:1	scorched flavor smooth texture
2/4	4.5	1.2	10.4	184.5	24.6	8.7:1	slightly bitter smooth, thick
2/5	4.4	1.2	5.3	130.8	21.2	4.4:1	nice flavor weak body
2/6	4.4	2.5	17.6	319.5	21.2	7:1	N.C.

N.C.=not checked

Table 5.3 Acidity (pH), gas liquid chromatographic analyses and comments on organoleptic characteristics of commercial buttermilk #3.

Sample (Brand/ batch)	pH	Acetaldeh. [ppm]	Diacetyl [ppm]	Acetoin [ppm]	Ethanol [ppm]	Diacetyl: Acetaldeh. ratio	Organoleptic comments
3/1	4.2	0.3	7.7	157.3	18.4	25.7:1	unclean flavor smooth texture
3/2	4.4	2.2	4.3	108.4	21.0	1.9:1	flat flavor weak body
3/3	4.3	1.6	3.9	159.5	19.6	2.4:1	flat flavor thick body
3/4	4.4	n.d.	2.5	172.8	28.9	–	clean flavor smooth, thick
3/5	4.3	n.d	5.1	151.4	26.6	–	unclean flavor
3/6	4.4	2.0	3.9	282.2	28.5	1.9:1	N.C.

n.d.=not detected; N.C.=not checked

Table 5.4 Acidity (pH), gas liquid chromatographic analyses and comments on organoleptic characteristics of commercial buttermilk #4.

Sample (Brand/ batch)	pH	Acetaldeh. [ppm]	Diacetyl [ppm]	Acetoin [ppm]	Ethanol [ppm]	Diacetyl: Acetaldeh. ratio	Organoleptic comments
4/1	4.3	4.2	9.5	209.1	26.5	2.3:1	scorched flavor weak body
4/2	4.3	n.d.	2.9	67.6	23.2	–	unclean flavor cheesy
4/3	4.3	2.5	n.d.	100.8	17.9	–	unclean,cheesy smooth texture
4/4	4.4	n.d.	n.d.	160.3	34.2	–	flat flavor smooth, thick
4/5	4.5	0.7	2.8	334.6	36.0	4:1	unnatural flavor
4/6	4.4	2.0	4.1	253.2	38.6	2:1	N.C.

n.d.=not detected; N.C.=not checked

Table 5.5 Acidity (pH), gas liquid chromatographic analyses and comments on organoleptic characteristics of commercial buttermilk #5.

Sample (Brand/ batch)	pH	Acetaldeh. [ppm]	Diacetyl [ppm]	Acetoin [ppm]	Ethanol [ppm]	Diacetyl: Acetaldeh. ratio	Organoleptic comments
5/1	4.5	1.7	3.9	90.9	41.2	2.3:1	curdy texture uneven body
5/2	4.5	3.7	3.0	76.2	33.6	0.8:1	unclean, bitter grainy body
5/3	4.5	n.d.	5.1	189.6	19.3	–	lot of gas curdy body
5/4	4.5	n.d.	7.4	208.9	64.4	–	salty smooth, thick
5/5	4.5	1.7	2.0	17.6	30.6	1.1:1	unnatural,sweet
5/6	4.4	1.6	1.2	164.6	53.5	0.7:1	N.C.

n.d.=not detected; N.C.=not checked

Table 5.6 Acidity (pH), gas liquid chromatographic analyses and comments on organoleptic characteristics of commercial buttermilk #6.

Sample (Brand/ batch)	pH	Acetaldeh. [ppm]	Diacetyl [ppm]	Acetoin [ppm]	Ethanol [ppm]	Diacetyl: Acetaldeh. ratio	Organoleptic comments
6/1	4.4	n.d.	1.1	336.7	37.0	–	flat flavor smooth body
6/2	4.6	2.6	0.8	145.7	30.50	0.3:1	flat flavor smooth texture
6/3	4.4	2.2	6.1	210.6	38.6	2.8:1	nice flavor smooth texture
6/4	4.5	n.d	n.d.	312.4	30.6	–	flat flavor smooth, thick
6/5	4.5	n.d	25.1	458.4	33.5	–	nice flavor slightly cooked
6/6	4.4	0.8	2.6	406.7	35.2	3.2:1	N. C.

n.d.=not detected; N. C.=not checked

The total MRS counts in the samples varied between $2.8x10^7$ and $1.4x10^9$ CFU/ml (Tables 5.7-5.9). All samples contained vancomycin resistant strains, presumably *Leu. cremoris,* as part of their starter mixture. Proportionally, vancomycin resistant species represented 0.3 to 25% of total MRS count in buttermilk samples, with buttermilk #1 being the lowest in vancomycin resistant counts. Green apple flavor, commonly found in samples of buttermilk #1, may be correlated with these results indicating an inadequate starter mixture.

Table 5.7 MRS plate counts of commercial buttermilks #1 and #2.

Sample	Vancomycin resistant count	MRS count	Sample	Vancomycin resistant count	MRS count
1/1	$10x10^5$	$22x10^7$	2/1	$22x10^6$	$36x10^7$
1/2	$71x10^5$	$82x10^6$	2/2	$10x10^7$	$47x10^7$
1/3	$21x10^5$	$22x10^7$	2/3	$28x10^6$	$11x10^7$
1/4	$10x10^5$	$25x10^7$	2/4	$46x10^6$	$76x10^7$
1/5	$10x10^5$	$76x10^6$	2/5	$64x10^6$	$72x10^7$
1/6	$17x10^5$	$38x10^7$	2/6	$24x10^5$	$28x10^6$

Table 5.8 MRS plate counts of commercial buttermilks #3 and #4.

Sample	Vancomycin resistant count	MRS count	Sample	Vancomycin resistant count	MRS count
3/1	$50x10^5$	$28x10^7$	4/1	$11x10^7$	$10x10^8$
3/2	$60x10^6$	$11x10^8$	4/2	$72x10^6$	$60x10^7$
3/3	$18x10^6$	$57x10^6$	4/3	$27x10^6$	$13x10^7$
3/4	$19x10^6$	$26x10^7$	4/4	$41x10^6$	$22x10^7$
3/5	$24x10^6$	$96x10^7$	4/5	$23x10^6$	$95x10^7$
3/6	$80x10^5$	$95x10^7$	4/6	$55x10^6$	$100x10^7$

Table 5.9 MRS plate counts of commercial buttermilks #5 and #6.

Sample	Vancomycin resistant count	MRS count	Sample	Vancomycin resistant count	MRS count
5/1	$28x10^6$	$20x10^7$	6/1	$22x10^6$	N.C.
5/2	$60x10^6$	$94x10^7$	6/2	$83x10^6$	$13x10^8$
5/3	$32x10^6$	$54x10^7$	6/3	$32x10^5$	$11x10^8$
5/4	$65x10^6$	$80x10^7$	6/4	$68x10^6$	$14x10^8$
5/5	$33x10^6$	$25x10^7$	6/5	$18x10^6$	$48x10^7$
5/6	$55x10^6$	$73x10^7$	6/6	$12x10^6$	$18x10^7$

Microbial quality of pasteurized, lowfat milks from six regional dairies was determined as CVT counts as an indication of post-pasteurization contamination. CVT counts were low except for milk #4 (730 CFU/ml) and #5 (2900 CFU/ml). Surprisingly, microscopic analyses of some isolates from CVT plates of samples 4 and 5 showed that the contaminants were Gram positive cocci. Isolates from milk sample #4 were thermoduric indicating contamination with *Streptococcus thermophilus*. Isolates from sample #5 did not

grow after 30 min pasteurization at 63°C. Although no further identification tests were applied, we considered the possibility that contaminants in sample #5 belonged to the *Enterococcus* genus.

Characteristics of experimental buttermilk

Results of GLC analyses and organoleptic evaluation of the four experimental buttermilks are shown in Table 5.10. All samples had delicate buttery flavor. Buttermilks made with *Leu. cremoris* 91404 and *Lc. cremoris* 205 (A and B) had a more distinct buttery flavor and were more carbonated than buttermilks C and D.

Table 5.10 Acidity (pH), gas liquid chromatographic analyses and comments on organoleptic characteristics of experimental buttermilks after four days of refrigeration.

Sample	pH	Acetald. [ppm]	Diacetyl [ppm]	Acetoin [ppm]	Ethanol [ppm]	Diacetyl: Acetald. Ratio	Organoleptic comments
A	4.6	0.9	3.4	179.3	174.2	3.8	buttery flavor slightly lumpy
B	4.7	0.8	2.1	95.4	162.3	2.6	buttery flavor high gas
C	4.6	1.2	3.6	220.0	150.5	3.0	nice flavor thick, shiny
D	4.6	n.d.	0.9	206.0	102.2	–	nice flavor thick, shiny

A=Lowfat milk inoculated with *Leu. cremoris* 91404 and *Lc.cremoris* 205;
B=Lowfat milk + citrate,0.1% inoculated with *Leu. cremoris* 91404 and *Lc.cremoris* 205;
C=Lowfat milk inoculated with *Leu. cremoris* 91404, *Lc.cremoris* 205 and 352;
D=Nonfat milk + citrate,0.1% inoculated with *Leu. cremoris* 91404, *Lc.cremoris* 205 and *Lc.cremoris* 352.

However, fortification of milk with citrate prior to pasteurization did not affect production and subsequent perception of flavor determinants (sample B *versus* sample A). Incorporation of ropy *Lc. cremoris* strain 352 in starter mixture resulted in texture improvement. Buttermilks C and D had thick, viscous and shiny bodies and very smooth

mouth feeling texture. The flavor of buttermilks C and D was very good, but somehow different from A and B. It will be interesting to investigate the interaction of polysaccharide, produced by the ropy strain, with milk components, and its possible effect on the flavor of buttermilk.

The effect of citrate, added during curd breaking, on flavor enhancement is illustrated on chromatograms of buttermilk C (Fig. 5.1 and 5.2). Immediately after fermentation there were no detectable amounts of diacetyl or acetoin in buttermilk C (Fig. 5.1). However, after four days of refrigeration concentrations of diacetyl and acetoin were 3.6 and 220 ppm, respectively (Fig 5.2). Obviously, fortification with citrate provided a new source of precursor from which diacetyl and acetoin were produced during cold storage.

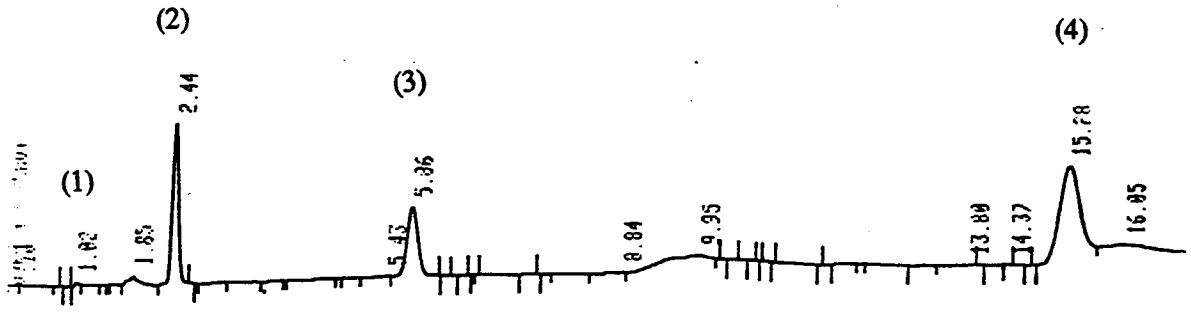

Figure 5.1 Chromatogram of experimental buttermilk C immediately after fermentation phase. GC peaks were identified as: (1) acetaldehyde, RT=1.02; (2) ethanol, RT=2.44; (3) sec-butanol (IS), RT=5.86; (4) acetic acid, RT=15.28. RT=retention time, min.

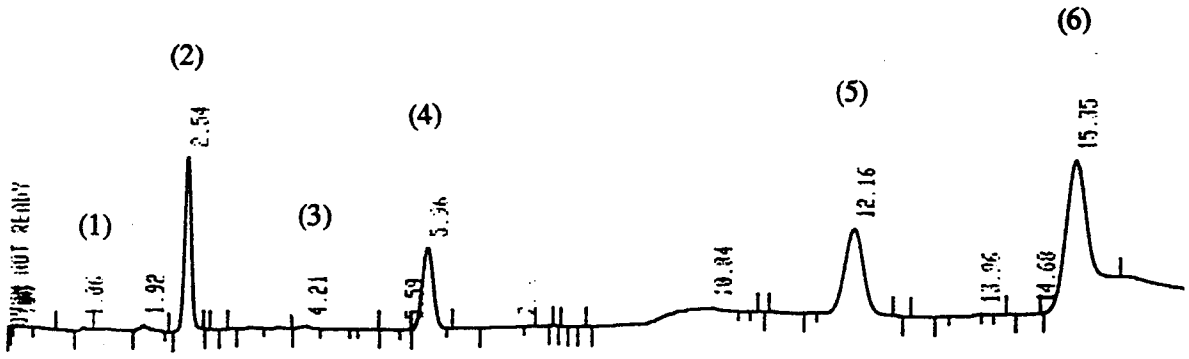

Figure 5.2 Chromatogram of experimental buttermilk C after four days of storage at 5°C. GC peaks were identified as: (1) acetaldehyde, RT=1.06; (2) ethanol, RT=2.54; (3) diacetyl, RT=4.21; (4) sec-butanol (IS), RT=5.96; (5) acetoin, RT=12.16; (6) acetic acid, RT=15.35. RT=retention time.

High quality buttermilk D was made from nonfat milk and could not be distinguished from buttermilk made from lowfat milk with same starter culture. *Leu. cremoris* 91404 provided nice flavor and ropy *L. cremoris* 352 provided excellent body for the buttermilk D. As we could not find any fat free buttermilk on the market, this product could be interesting for the consumers concerned about fat content of the dairy products.

The procedure for production of high quality buttermilk is summarized as follows (Levata-Jovanovic and Sandine, 1994b):

1. Select high quality lowfat milk (1-1.5%).
2. Fortify to 9.0% solids-non-fat with NDM to help improve body and texture.
3. Add 1 pound of sodium citrate per 100 gallons of milk (optional).
4. Add salt at rate of 7 to 8 lb per 100 gallons of milk.
5 Batch pasteurize the milk to 185°F for 30-45 min
6. Thaw two cans of *Lactococcus cremoris* (acid producer) and two cans of *Leuconostoc cremoris* 91404 (flavor producer) in cool chlorinated water.
7. Add all four cans to set 300-1,000 gallons of milk.
8. Agitate the milk slowly for 5 min to thoroughly mix in the cultures.
9. Incubate the inoculated milk at 72°F, without agitation, until a titrable acidity of 0.8-0.85% (pH 4.6-4.7) is reached. This will take approximately 16-18 hours.
10. Turn on the cooling water and gently agitate the coagulum to break it. Immediately add a previously prepared sodium citrate solution* to a final level of 0.2% (2lbs sodium citrate per 100 gallons milk).
11. Stir at slow speed until buttermilk is cooled to 40°F and package.

*Prepare sodium citrate solution by dissolving 2 lb of sodium citrate in 0.5 to 1 gallon of water in a Pyrex or stainless steel container. Autoclave the solution for 10 min at 250°F. Cool and add to cultured buttermilk.

5.4 Conclusions

1. A survey of commercial buttermilks available in Corvallis, Oregon, showed wide variation in flavor score and in concentrations of acetaldehyde, diacetyl, acetoin, ethanol and acetic acid.

2. In order to make high quality, flavorful buttermilk it was suggested to introduce one more step in traditional procedure for manufacturing cultured buttermilk. This step consists of fortification with citrate during coagulum breaking to provide substrate for diacetyl/acetoin production by *Leu. cremoris* 91404 during refrigeration.

3. Use of ropy, polysaccharide-producing *Lc. cremoris* strain, as acid producer in starter mixtures, significantly improved the texture and body of the buttermilk.

4. Nonfat, slightly aromatic, clean, thick, shiny, carbon dioxide containing buttermilk was made with *Leu. cremoris* 91404 and *Lc. cremoris* 205 and 352 as starters using a recommended procedure.

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